DOCTOR OF PHILOSOPHY

The influence of Vitamin D on in vivo immunity

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Title: The influence of vitamin D on *in vivo* immunity

By

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Supervisor(s) Dr. Samuel J. Oliver & Prof. Neil P. Walsh

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Summary

In order to assess the influence of vitamin D on *in vivo* immunity, there is a need for well-controlled field studies and randomised placebo-controlled trials to be conducted among young healthy adults, using clinically relevant *in vivo* measures of immune competence. The broad aim of this thesis was to investigate the influence of vitamin D on the development of *in vivo* immunity, as assessed by the secondary antibody response to hepatitis B vaccination (anti-HBs). Firstly, in a prospective cohort study of 447 healthy young men and women, we examined the influence of vitamin D status (serum 25(OH)D) on secondary hepatitis vaccination response. We demonstrated that fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations. These associations were particularly stronger in men compared to women, likely due to a greater prevalence of vitamin D insufficiency in men compared to women (serum 25(OH)D ≥ 50 nmol/L; men *vs.* women, 49% *vs.* 70%). These findings may in part explain the observed seasonal variation in secondary hepatitis B vaccination response, in that fewer men and women were vaccine responders in the winter compared to summer (44% *vs.* 62%, respectively). This seasonal variation in secondary hepatitis B vaccination response also mirrored seasonal variation in serum 25(OH)D and $1,25(OH)_2D$ concentrations as well as the higher prevalence of vitamin D insufficiency during the winter (**Chapter 4**). Then we demonstrated that simulated sunlight and oral vitamin D_3 supplementation restored 25(OH)D from winter concentrations to concentrations typical during summer within 4 weeks and maintained IOM and EFSA vitamin D sufficiency (serum 25(OH)D ≥ 50 nmol/L) concentrations for a further 8 weeks (**Chapter 5**). After demonstrating that the change in serum 25(OH)D was matched between simulated sunlight and oral vitamin D_3 (**Chapter 5**), we applied these methods to a randomised placebo-controlled trial that investigated the effect of 12-weeks vitamin D supplementation on the secondary hepatitis vaccination response in 119 healthy young men. Results showed that vitamin D supplementation, via simulated sunlight and oral vitamin D_3 supplementation did not influence secondary anti-HBs response (**Chapter 6**). Future studies should seek to raise vitamin D status prior to initial vaccination; using methods that replicate safe, practical and government recommended summer sunlight and oral vitamin D_3 supplementation guidelines.

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Publications

For all research chapters I was involved in the data collection, data analyses and preparation of material for publication. In addition, for **Chapters 4-6** I was also involved in all aspects of the study design. The following list shows the publications that have been produced from the material presented in this thesis.

The following list shows the publications that have been produced because of work undertaken as part of a wider MoD research project, but not presented here.

Full papers

Carswell, AT., Oliver, SJ., Wentz, LM., Kashi, DS., Roberts, R., Tang, JCY., Izard, RM., Jackson, S., Allan, D., Rhodes, LE., Fraser, WD., Greeves, JP., Walsh, NP., 2018. Influence of vitamin D supplementation by sunlight or oral D_3 on exercise performance. Med. Sci. Sports Exerc. [epub ahead of print]. DOI: 10.1249/MSS.000000000000172.

Abstracts

Carswell, AT., Wentz, LM., Oliver, SJ., Kashi, DS., Potter, C., Ward, MD., AW Yong, XH., Tang, JCY., Jackson, S., Izard, RM., Fraser, WD., Greeves, JP., Walsh, NP. 2017. Vitamin D status is an important predictor of aerobic performance in male and female Army recruits. International Journal of Sport Nutrition and Exercise Metabolism. 27, S1-S20.

Conference proceedings

Kashi, DS. 2016. Vitamin D sufficiency improves hepatitis B vaccine response in male Army Recruits. Verbal presentation at ISENC, Newcastle-upon-Tyne, UK, December 2016.

Wentz, LM. 2017. Military Recruits Who Typically Sleep <6 hours miss training due to Upper respiratory infection. Poster presentation at the ACSM Annual Meeting, Denver, CO, USA, May 2017.

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List of Abbreviations

1, 25$(OH)_2D_3$	1, 25-dihydroxyvitamin D
24, 25$(OH)_2D$	24, 25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
AMPs	antimicrobial proteins
anti-HBs	hepatitis B surface antibody
ANOVA	analysis of variance
APCs	antigen presenting cells
BMI	body mass index
CYP24A1	24, 25 hydroxylase
CYP27B1	1-α hydroxylase
DCs	dendritic cells
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
IgG	immunoglobulin-G
IL	interleukin
IOM	Institute of Medicine
IU	International units
L	litre
LC-MS/MS	liquid chromatography-tandem mass spectroscopy
μg	microgram
MED	minimal erythemal dose
MMR	measles mumps and rubella
OPV	oral polio virus
ORAL	oral vitamin D_3
PTH	parathyroid hormone
SD	standard deviation
SED	standard erythemal dose
SIgA	secretory immunglobulin-A
SSR	simulated solar radiation
TCR	T cell receptor
TLRs	toll-like receptors

UK	United Kingdom
URI	upper respiratory illness
URTI	upper respiratory tract infection
UVB	ultraviolet B
UVR	ultraviolet radiation
Vitamin D_3	cholecalciferol
Vitamin D_2	ergocalciferol
VDBP	vitamin D binding protein
VDRE	vitamin D response elements

CHAPTER ONE
General Introduction

Vitamin D was first identified in the early twentieth century by the work of nutritional biochemist MccCollum. McCollum's pioneering work on experimental rickets was the first to identify the existence of a vitamin that was responsible for calcium deposition (McCollum, Simmonds, & Becker, 1922), later called vitamin D. As a result of his work, and the rickets epidemic of the same era, vitamin D was long recognised only for its role in bone health. Studies that followed determined the main source of vitamin D as ultraviolet B (UVB) radiation exposure and showed that limited quantities could be obtained from the diet alone (Holick, 2002). Consequently, serum concentrations of 25-hydroxyvitamin D (25(OH)D, a marker of nutritional vitamin D status) demonstrate strong seasonal patterns, falling to a nadir during the winter and peaking during summer. Specifically, over the 6 month winter and spring period (October-April) all of Scandinavia, most of western Europe (including 90% of the UK) and 50% of North America lies above the latitude that permits exposure to the ultraviolet B wavelength necessary for vitamin D synthesis. In the United Kingdom alone, more than 50% of the adult population display insufficient vitamin D status (serum 25-hydroxyvitamin D (25(OH)D) < 50 nmol/L) and 16% have severe deficiency during the winter and spring (Pearce & Cheetham, 2010).

During the past decades, knowledge of the vitamin D endocrine system and its biological significance has grown exponentially. Generation of a vitamin D receptor knockout mouse (Li et al., 1997) and high throughput gene microarrays have identified vitamin D targets in numerous tissues such as bone (Ebeling, 2014; Zarei, Morovat, Javaid, & Brown, 2016), immune system cells (Baeke, Takiishi, Korf, Gysemans, & Mathieu, 2010), the cardiovascular system (Lavie, Dinicolantonio, Milani, & O'Keefe, 2013), and skeletal muscle (Hamilton, 2010). Thus, avoiding low serum 25(OH)D is essential for multiple health outcomes (Bischoff-Ferrari, 2014), with current Institute of medicine (IOM) and European Food Safety Authority (EFSA) recommendations to maintain serum 25(OH)D concentration ≥ 50 nmol/L (European Food Safety Authority, 2016; Institute of Medicine, 2011).

The discovery of the vitamin D receptor (VDR) in almost all immune cells, and the variety of roles vitamin D has in both the innate and adaptive arms of immunity *in vitro* (Chang, Chung,

& Dong, 2010; He, Handzlik, et al., 2013; Liu et al., 2006; Wang et al., 2004), demonstrate the importance of vitamin D in the regulation of immune response (Baeke et al., 2010). Indeed, large cross sectional studies and a recent meta-analysis of vitamin D supplementations have shown the importance of vitamin D to immune health (Berry, Hesketh, Power, & Hypponen, 2011; Ginde, Mansbach, & Camargo, 2009; Martineau et al., 2017). However, previous studies, for convenience, have tended to rely on self-report of URI, using common cold symptom questionnaires (Berry et al., 2011; Ginde et al., 2009; He, Handzlik, et al., 2013), which have received some criticism (Cox et al., 2008). Furthermore, in an attempt to understand the mechanisms underpinning the influence of vitamin D on aspects of innate and adaptive immune function, researchers have relied on *in vitro* based measures of immune function, which typically involves isolated analysis of immune cells in artificial environments (Akbar et al., 2013).

The importance and need for studies examining the influence of seasonal changes in vitamin D status and vitamin D supplementation on *in vivo* measures with known clinical endpoints, such as the antibody response to vaccinations, has recently been highlighted (He, Aw Yong, Walsh, & Gleeson, 2016). Vaccinations induce a multistep immune response orchestrated by the initial processing of the antigen at the site of vaccination and the eventual development of antigen specific memory T and B cells as effectors of immune protection. The measurement of serum antibody status following vaccination represents the culmination of a series of interactions of different immune cell types in various immune compartments. Therefore, the measurement of antibodies following vaccination provides an accurate reflection of the functional status of the immune system *in vivo.* Animal research suggests that the critical stages of orchestrating the development of new immunity, termed induction, are most sensitive to immune impairment (Fleshner et al., 1992; Okimura, Ogawa, & Yamauchi, 1986). Limited studies in humans show similar findings, where stress in close proximity to the first immune challenge has an immune modulatory effect (Harper Smith et al., 2011), but stress close to the recall has a greatly reduced effect (Harper Smith et al., 2011) or no effect (Smith et al., 2004) on the development of new *in vivo* immunity.

Interpreting the findings of cross sectional and supplementation studies investigating the influence of vitamin D status on *in vivo* antibody response following vaccination, are challenging. Though one study demonstrates lower antibody response following vaccination among chronic kidney disease patients with low vitamin D status (Zitt, Sprenger-Mahr, Knoll,

Neyer, & Lhotta, 2012), studies are confounded by participants being elderly and diseased (Zitt et al., 2012), vitamin D sufficient at baseline (Sundaram et al., 2013) or previous exposure to vaccine antigens (Kriesel & Spruance, 1999). Further, it is not well understood if vitamin D status is important for initial capturing and processing of an antigen at the site of vaccination or the subsequent downstream effects leading to the development of immune memory. Therefore, the broad aim of this thesis was to investigate the influence of vitamin D on the development of *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccine (anti-HBs).

CHAPTER TWO

Literature Review

2. 1 Vitamin D structure and sources

Vitamin D belongs to a family of lipids called secosteroids, which are very similar in structure to steroids with the exception that two of the B-ring carbon atoms of the typical four steroid rings are not joined, whereas in steroids they are. The biologically active metabolite form of vitamin D, 1,25-dihydroxyvitamin D (1, $25(OH)_2D$), acts very much like a steroid binding to nuclear receptors and modulating gene expression and subsequently the synthesis of specific proteins. The two forms of vitamin D, vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol) can be obtained from dietary sources. While vitamin D_3 is found in food from animal origin, including; oily fish, egg yolk, liver and milk, vitamin D_2 is present in some plants and mushrooms (derived from ultraviolet B (UVB) exposure to fungi and yeast ergosterols). Some food including cereals, margarine and dairy products are fortified, usually with vitamin D_3. However, the major source of vitamin D, providing 80-100% of the body's vitamin D requirements, occurs as a result of UVB mediated conversion of 7 dehydrochloesterol in the plasma membrane of cells through short lasting skin exposures to natural UVB irradiation from summer sunlight (~15 minutes each day) (Webb, Kline, & Holick, 1988). It should be noted that prolonged exposures give diminishing returns in terms of vitamin D formation (Holick, 1995) and in fact heighten the risk of erythema (i.e. sunburn) for fairer skin types, and more severely; skin cancer (Armstrong & Kricker, 2001).

2.1.1 Vitamin D metabolism

A primary step leading to the cutaneous synthesis of pre-vitamin D_3 is the absorption of UVB protons (290-315nm) by 7-dehydrocholesterol at the skin surface, which is subsequently converted into vitamin D_3, by a spontaneous thermal isomerisation. Newly synthesized vitamin D_3 (and its metabolites) are bound to vitamin D-binding protein (VDBP) for systemic transport. In order to become the biologically active 1, $25(OH)_2D$, vitamin D needs to undergo two hydroxylation steps. Whether made in the skin or ingested in the diet, pre-vitamin D (calcidiol or calcifediol) is transported to the liver where it undergoes hydroxylation to form 25(OH)D, at the carbon 25 position by the enzyme 25-hydroxylase. This main storage form of vitamin D is found in muscles and adipose tissue and is the major circulating form of vitamin D 25(OH)D. Subsequently 25(OH)D is converted to the biologically active form, 1, $25(OH)_2D$ in a second hydroxylation step in the kidneys. This hydroxylation occurs due to the enzyme 1-α

hydroxylase, an enzyme which is stimulated by parathyroid hormone (PTH) when calcium and phosphate concentrations fall below their normal physiological range, 2.1-2.6 mmol/L and 1.0-1.5 mmol/L respectively (He, Aw Yong, et al., 2016). The biologically active 1, $25(OH)_2D$, is released into the circulation from the kidney which is considered as a vital endocrine source of hormone. Normal concentrations of circulating $1,25(OH)_2D$ are approximately 50-250 pmol/L, about 1000 times lower than its precursor, 25(OH)D; and the plasma half-life of 1, $25(OH)_2D$ is 4-6 hours. It is important to acknowledge that 1, $25(OH)_2D$ limits its own activity in a negative feedback loop through the induction of 24 hydroxylase, which coverts 1, $25(OH)_2D$ to the biologically inert metabolite 24, $25(OH)_3D$. Additionally, 1, $25(OH)_2D$ also inhibits the expression of renal 1-α hydroxylase. This negative feedback loop reduces the likelihood of hypercalcaemia by preventing excessive vitamin D signalling, this maintaining bone health (Owens et al., 2017).

2.1.2 Vitamin D status

Although it would seem intuitive that measurement of 1, $25(OH)_2D$ would be a suitable indicator of vitamin D status. Under conditions of low vitamin D status, the subsequent elevation in PTH enhances renal 1-α-hydroxylase activity which promotes conversion of available 25(OH)D to 1, $25(OH)_2D$. Therefore, even in settings of low vitamin D, 1, $25(OH)_2D$ may be maintained within the normal range. Secondly, 1, 25 $(OH)_2D$ has a relatively short half-life (< 4 hours) and its concentrations are ~1000 times lower than its precursor 25(OH)D. The inactive but major circulating form of vitamin D, 25(OH)D, is regarded as the appropriate index of a human's vitamin D nutritional status by the Food and Nutrition board of the institute of medicine. With a reported half-life of 2 to 3 weeks (Baeke et al., 2010), 25(OH)D concentrations are much more stable than 1, $25(OH)_2D$. From a practical perspective, it has also been demonstrated that 25(OH)D in whole blood, serum or plasma is stable at room temperature or when stored at -20°C and is unaffected by multiple freeze thaw cycles (Antoniucci, Black, & Sellmeyer, 2005). Additionally, 25(OH)D is also used as a substrate for most extra renal tissues, which have the capacity to synthesize 1, $25(OH)_2D$ for their own use, but depend on adequate serum concentrations of 25(OH)D (Aranow, 2011).

2.1.3 Vitamin D classifications and boundaries

There still remains considerable controversy about what 25(OH)D concentrations defines deficiency, insufficiency and sufficiency. However, to maximise vitamin D's effects on

calcium metabolism the IOM has recommended 25(OH)D plasma concentrations ≥ 100nmol/L are defined as optimal, 50 to 100 nmol/L are defined as sufficient, and values < 30 nmol/L represent vitamin D deficiency in reference to achieving 'good bone health' (Institute of Medicine, 2011). However, the Endocrine society and a number of world leading researchers on the field propose that concentrations < 50 nmol/L should be regarded as deficient (Bischoff-Ferrari, 2014). Indeed, a comprehensive review summarising various studies that have attempted to evaluate threshold concentrations of serum 25(OH)D status in relation to bone mineral density, lower limb function, dental health, cancer prevention, risk of falls, fractures, incident hypertension and mortality concludes that for all endpoints, serum concentrations of 25(OH)D < 50 nmol/L are associated with adverse effects or no benefit (Bischoff-Ferrari, 2014). This lack of definitive consensus of opinion on the threshold for vitamin D status and bone health is potentially fuelled by the variation in 25(OH)D measurement methodology.

2.1.4 Measuring Vitamin D status

As a result of the high prevalence of vitamin D insufficiency (25(OH)D < 50 nmol/L, reviewed in section 2.2) and discoveries regarding vitamin Ds non classical functions beyond bone health (He, Aw Yong, et al., 2016; Owens, Fraser, & Close, 2015). There have been growing demands for the measurement of vitamin D (He, Gleeson, & Fraser, 2013). No one method has been deemed the reference assay; however, a "physico-chemical" method such as the liquid chromatography-tandem mass spectroscopy (LC-MS/MS) represents one potential candidate. Recently, researchers have sought to utilize commercial ELISAs to assess 25(OH)D in order to circumnavigate the need for expensive equipment, large plasma sample volumes and the requirement for specialist staff which the LC-MS/MS method demands. Current 25(OH)D ELISAs employ polyclonal or monoclonal antibodies that bind specifically to human 25(OH)D. However, the competition between the 25(OH)D specific antibody and the VDBP in plasma samples makes these assays difficult to control (Chen et al., 2010; Farrell et al., 2012). The plasma 25(OH)D concentration cannot be measured accurately unless it is released from the VDBP and the strong protein binding of 25(OH)D requires the employment of suitable conditions to release 25(OH)D from VDBP (Fraser & Milan, 2013). Furthermore, most commercial immunoassays, cannot measure the concentrations of 25(OH)D_2 and 25(OH)D_3 independently, with some evidence demonstrating an underestimation of 25(OH)D_2 in several commercial immunoassays, resulting in marked variations of the total plasma/serum 25(OH)D concentration (D_2 and D_3). In contrast, the LC/MS/MS method is regarded as the gold standard for the measurement of serum 25(OH)D concentration, because it can separate and accurately

quantify both 25(OH)D_2 and 25(OH)D_3; additionally, an extraction procedure ensures that both free 25(OH)D and protein bound 25(OH)D are quantified (Wallace, Gibson, de la Hunty, Lamberg-Allardt, & Ashwell, 2010).

2.2 Prevalence of vitamin D insufficiency

Despite the controversy about what 25(OH)D concentrations defines deficiency, insufficiency and sufficiency, both the Institute of Medicine (IOM) and European Food Safety Authority (EFSA) recommend to maintain serum 25(OH)D concentration ≥ 50 nmol/L (European Food Safety Authority, 2016; Institute of Medicine, 2011). However, it is estimated that 1 billion people worldwide have vitamin D status below this threshold, making vitamin D insufficiency one of the most common medical conditions in the world (Holick, 2007). As logic dictates, vitamin D status is significantly compromised during winter months at latitudes above 35°, when skin sunlight UVB exposure and endogenous synthesis is low. Specifically, over the 6 month winter and spring period (October – April) all of Scandinavia, most of western Europe (including 90% of the UK) and 50% of North America lies above the latitude that permits exposure to the UVB wavelength necessary for vitamin D synthesis (Pearce & Cheetham, 2010). In the United Kingdom alone, more than 50% of the adult population display insufficient vitamin D status and 16% have severe deficiency during the winter and spring (He, Aw Yong, et al., 2016). Vitamin D insufficiency is particularly prevalent among the elderly. Biologically this is due to the decrease in the amount of 7-dehydrocholersterol available in the epidermal layer of the skin, where the majority of vitamin D is formed following exposure to UVB radiation (MacLaughlin & Holick, 1985). High prevalence of vitamin D insufficiency among elderly can also be explained by behavioural tendencies of spending long periods indoors and wearing more clothing. However, it should also be acknowledged that behavioural tendencies, season and latitude of residence, as well as genetic disposition may also help explain why high rates of vitamin D insufficiency are also observed in young and otherwise healthy adult populations. Indeed, during winter months more than half of this population can be considered vitamin D insufficient and as many as 35% deficient. A summary of the current evidence on vitamin D status in young, healthy adults is provided in table 2.1.

2.2.1 Reasons for vitamin D insufficiency: Genetic and biological differences

Firstly, the effectiveness of cutaneous synthesis of vitamin D is determined by skin pigmentation, because melanin efficiently absorbs UVB radiation (Macdonald, 2013). For instance, both lower cutaneous synthesis of pre-vitamin D in darker human skin samples, and

lower 25(OH)D responses to repeated UVB radiation demonstrate the importance of skin pigmentation in the cutaneous synthesis of vitamin D (Chen et al., 2007). Recent evidence also indicates that racial differences in manifestation of vitamin D status may be related to genetic variations in vitamin D binding protein (VDBP). VDBP is the primary vitamin D carrier, binding 85-90% of serum 25(OH)D and 1, $25(OH)_2D$ and the remaining unbound 25(OH)D is considered bioavailable (either free or bound to albumin). About 10-15% of total 25(OH)D is bound to albumin, in contrast to free 25(OH)D which accounts for < 1% of serum vitamin D (Bikle et al., 1986). Since the affinity of albumin to 25(OH)D or 1, $25(OH)_2D$ is weaker than that of the VDBP, the loosely bound fraction comprise bioavailable 25(OH)D (Brown & Coyne, 2012). Genotyping has identified two common single nucleotide polymorphisms (SNPs) in the coding region of the VDBP gene (rs4588 and rs7041) (Malik et al., 2013). Combinations of these two SNPs produce three major polymorphioc forms of VDBP which differ substantially in their binding affinity for 25(OH)D, serum concentrations, and variation between ethnic groups. These factors are in turn linked to VDBP function. These variants change the amino acid sequence, alter the protein function, and are common enough to generate population wide constitutive differences in vitamin D status (Powe et al., 2011).

2.2.2 Reasons for vitamin D insufficiency: Behavioural and lifestyle

Because of the concern about skin cancer, many are now encouraged to avoid direct sunlight, by seeking shade and applying sunscreen, limiting the amount of UVB reaching the skin. The use of sunscreen interferes with vitamin D_3 formation by absorbing and reflecting UVB radiation, thus preventing UVB radiation from reaching the target skin cells. Indeed, topical application of a sunscreen with a sun protection factor of 8 has been found to limit vitamin D_3 production in protected compared to unprotected participants (Matsuoka, Ide, Wortsman, Maclaughlin, & Holick, 1987). Further, sunscreen with sun protection factor 15 or more blocks ~99% of dermal vitamin D synthesis (Webb, 2006). Clothing can also act as a physical barriers preventing UVB from reaching the skin. Broadly speaking, any area covered by clothing will reduce the exposed skin surface area to sunlight. At particular risk are individuals who wear standardised dress bound to religion and cultural beliefs (headscarves and vails), or occupation (military personnel). Furthermore, long hours spent indoors is not only restricted to the elderly as it is now common place that younger adults have jobs confined to offices and long hours spent at a desk, limiting the exposure to natural UVB.

2.3 Biological role of vitamin D for bone health

McCollum's pioneering work on experimental rickets was the first to identify the existence of a vitamin that was responsible for calcium deposition (McCollum et al., 1922), later called vitamin D. As a result of his work, and the rickets epidemic of the same era, vitamin D was long recognised only for its role in bone health. Vitamin D influences bone health by upregulating the expression of genes for several calcium transport proteins that enhance calcium absorption from the diet in the small intestine and increase calcium reabsorption in the renal tubules (in association with elevated PTH) (Holick, 2007). When dietary calcium is inadequate to satisfy the body's calcium requirement, 1, 25$(OH)_2$D in concert with PTH, mobilise monocytic stem cells in the bone marrow to become mature oseteoclasts, which in turn enhances the removal of calcium from the bone into the circulation to maintain normal serum calcium concentrations (Holick, 2007). In the general population, the role of vitamin D with calcium homeostasis and bone health is exemplified by higher bone mineral density in the hips and lumbar spine among those with higher vitamin D status (Holick, 2007).

During the past decades, knowledge of the vitamin D endocrine system and its biological significance has grown exponentially. Generation of a vitamin D receptor knockout mouse (Li et al., 1997) and high throughout gene microarrays have identified vitamin D targets in numerous tissues such as bone (Ebeling, 2014; Zarei et al., 2016), immune system cells (Baeke et al., 2010), the cardiovascular system (Lavie et al., 2013), and skeletal muscle (Hamilton, 2010). Thus, avoiding low serum 25(OH)D is essential for multiple health outcomes (Bischoff-Ferrari, 2014), with current Institute of medicine and European Food safety Authority (EFSA) recommendations to maintain serum 25(OH)D concentration $\geq$ 50 nmol/L (European Food Safety Authority, 2016; Institute of Medicine, 2011).

Table 2.1. Vitamin D status in young healthy adults, adapted from **(He, Aw Yong, et al., 2016)**.

Season	Reference	Location	Population	N	Age	Serum 25(OH)D concentration (nmol/L)		
						% < 30 nmol/L Deficient	**% < 50 nmol/L Insufficient**	**% < 75 mol/L Suboptimal**
Winter								
	Laaksi et al	Finland (60-70° N)	Finnish Military recruits	196	18 – 28	19% < 25	78% < 40	-
	Morton et al	Liverpool, UK (53° N)	Elite Soccer players	20	24	-	65%	-
	Close et al	Liverpool, UK (53° N)	UK club athletes	30	20 – 24	20%	57%	-
	Close et al	Liverpool, UK (53° N)	Professional UK athletes	61	18 – 27	35%	64%	-
	He et al	Loughborough, UK (53° N)	Recreational to elite athletes	225	21	8%	38%	-
	Garcia & Guisado	Barcelona 41° N)	Professional basketball players	21	25	10% < 25	57%	-
Autumn								
	Storlie et al	Washington, USA (47° N)	Collegiate athletes	39	18 – 33	-	3%	34% < 80
	Lovell	Australia (35° S)	Australian female gymnasts	18	10 – 17	-	33%	83%

Season	Reference	Location	Population	N	Age	Serum 25(OH)D concentration (nmol/L)		
						% < 30 nmol/L Deficient	**% < 50 nmol/L Insufficient**	**% < 75 mol/L Suboptimal**
Summer						-		-
	Laaksi et al	Finland (60-70° N)	Finnish military	756	18 – 29	-	4% < 40	-
	Villacis et al	California, USA (34° N)	Collegiate athletes	223	-	-	3%	34% < 80
	Hamilton et al	Doha, Qatar (25° N)	Middle eastern sportsmen	93	13 – 45	59% < 25	91%	100%
	Hamilton et al	Doha, Qatar (25° N)	Professional Qatar based footballers	342	16 – 33	12% < 25	56%	84%
All seasons								
	Wentz et al	Carolina, USA (35° N)	Young active military personnel	312	-	-	-	52%

Age is presented as mean or range. Hyphen indicates not reported.

2.4 Vitamin D and the immune system

The discovery of the VDR in almost all immune cells, including T-lymphocytes, B-lymphocytes, neutrophils and APCs, such as monocytes and macrophages and dendritic cells prompted the idea that vitamin D could have a vital role in the regulation of immune response and therefore host defence (Baeke et al., 2010). These immune cells have the capacity to express the mitochondrial vitamin D activating enzyme, 1-α-hydroxylase (CYP27B1) and thus possess ability to convert 25(OH)D to 1, 25$(OH)_2$D. This conversion is regulated by the circulating concentrations of 25(OH)D and can also be induced by activations of specific toll like receptors (TLRs) which act as pathogen detectors. Thus 1, 25$(OH)_2$D could play important roles in both innate and adaptive immune responses. Mechanisms by which vitamin D may influence immune function are; first via its direct endocrine actions on immune cells mediated by 1, 25$(OH)_2$D formed in the kidney. Secondly, direct intracellular actions of 1, 25$(OH)_2$D following intracrine conversion of 25(OH)D to 1, 25$(OH)_2$D within immune cells. Thirdly, through paracrine actions of 1, 25$(OH)_2$D produced in and secreted from APCs on local lymphocytes and neutrophils. Lastly, through the indirect actions on antigen presentation on T-cells mediated by 1, 25$(OH)_2$D on APCs. Several *in vitro* studies have elucidated vitamin D's actions on innate and adaptive immune cells; providing evidence for the physiological role of vitamin D in immunity.

2.4.1 Vitamin D and innate immune function

It has been demonstrated that 1, 25$(OH)_2$D is a vital mediator of innate immune responses, enhancing the antimicrobial properties of immune cells such as monocytes and macrophages through the induction of antimicrobial proteins (AMPs) and stimulation of autophagy and autophagosome activity (Bikle, 2009). Antimicrobial proteins, such as cathelicidin and β-defensin, are produced by epithelial cells and macrophages; and in the lungs are secreted into the biofilm covering the inner surface of the airways, thereby creating a barrier that is chemically lethal to microbes. 1, 25$(OH)_2$D is a key link between TLR activation and AMPs response in innate immunity. Following activation of the TLR signalling cascade in the presence of microbes, 1, 25$(OH)_2$D has a vital role in upregulating the production of AMPs (Wang et al., 2004). Both macrophages and epithelial cells, possessing the 1-α-hydroxylase and VDR are capable of responding to and producing 1, 25$(OH)_2$D.

1, 25$(OH)_2$D can induce expression of the vitamin responsive genes to enhance the production of cathelicidin and β-defensin by binding to vitamin D response elements (VDREs). The

stimulation of TLRs by interaction with pathogen-associated molecular patterns (PAMPs) in macrophages results in increased expression of both the VDR and the 1-α-hydroxylase enzyme, which upregulates the production of 1, 25$(OH)_2$D to stimulate the expression of cathelicidin and β-defensins (Liu, Stenger, Tang, & Modlin, 2007). In cell viability assays of macrophages infected with *M. tuberculosis*, increasing concentrations of cathelicidin resulted in the killing of intra-cellular *M. tuberculosis* (Liu et al., 2006). However, when human sera from vitamin D deficient African American participants was added to human macrophage cultures no upregulation of cathelicidin was detected. When sera was subsequently supplemented with 1, 25$(OH)_2$D, cathelicidin response was restored (Liu et al., 2006). Therefore, authors proposed that the stimulation of TLRs by interaction with specific PAMPs, in this instance *M. tuberculosis*, results in the upregulation of vitamin D machinery, namely the VDR and the 1-α-hydroxylase enzyme, which leads to enhanced cathelicidin production only in the presence of adequate vitamin D status, 25(OH)D (Liu et al., 2006).

2. 4. 2 Vitamin D and mucosal immunity

Further studies in animals and young healthy adults show benefits of vitamin D on both innate and mucosal immunity. Animal studies have demonstrated that VDRs are present in the parotid, submandibular and sublingual salivary glands which points to a possible role for vitamin D in the regulation of salivary secretion. This is supported by the finding that salivary flow rates were stimulated after treatment with vitamin D_3, in vitamin D deficient rats (Peterfy, Tenenhouse, & Yu, 1988). One study conducted in humans, has shown that in a cohort of university athletes, higher concentrations of plasma cathelicidin and salivary immunoglobulin A (SIgA) secretion were observed among those who had plasma 25(OH)D ≥ 120 nmol/L compared to those with lower vitamin D status (He, Handzlik, et al., 2013). In a subsequent follow-up randomised placebo-controlled, double-blind vitamin D_3 supplementation study (5,000 IU/day for 14 weeks), increases in salivary secretion rates of both SIgA and cathelicidin were observed in vitamin D_3 supplemented individuals, compared with no significant changes in a matched placebo group (He, Fraser, et al., 2016). This was in part explained by a significant increase in saliva flow rates over time in the vitamin D_3 supplemented group. Vitamin D might stimulate salivary secretion through the regulation of calcium. The rapid reflux of calcium plays a role in the stimulation of fluid secretion and potentially forms a candidate mechanism by which vitamin D affects saliva flow rate (Peterfy et al., 1988)

2.4.3 Vitamin D and adaptive immune function

In contrast with the innate immune responses, many of the reported actions of vitamin D on adaptive immunity are indicative of a more tolerogenic or anti-inflammatory immune status. Early studies investigating the influence of vitamin D on human adaptive immune cells demonstrated an expression of the VDR as well as vitamin D activating enzymes in both T-and B cells (Provvedini, Tsoukas, Deftos, & Manolagas, 1983). Under resting conditions, VDR expression by these cells is low, but upon activation and proliferation, T- and B cells upregulate VDR expression significantly facilitating the regulation of up to 500 vitamin D responsive genes which influence the differentiation and proliferation of these cells (S. Chen et al., 2007; Lemire, Adams, Sakai, & Jordan, 1984; Mahon, Wittke, Weaver, & Cantorna, 2003).

Firstly, T cells are thought to be an important target for the immunomodulatory effects of different forms of vitamin D. Four potential mechanisms by which vitamin D may influence T cell function include; (1) direct endocrine effects on T cells mediated via systemic calcitriol (1, 25(OH) $_2$D), (2) direct intracrine conversion of 25(OH)D to 1, 25(OH) $_2$D by T cells, (3) direct paracrine effects of 1, 25(OH) $_2$D on T cells following conversion of 25(OH)D to 1, 25(OH) $_2$D by monocytes or DCs, (4) indirect effects on antigen presentation on T cells mediated via localised APC affected by 1, 25(OH) $_2$D (Prietl, Treiber, Pieber, & Amrein, 2013). 1,25(OH) $_2$D can inhibit T-cell proliferation and also influence the phenotype of T-cells through the suppression of Th1 cells by inhibiting the production of IL-2 and interferon gamma (IGN-γ) that are associated with cellular immunity (Muller, Odum, & Bendtzen, 1993; Rigby, Stacy, & Fanger, 1984; Tsoukas et al., 1989). In contrast, IL-4, IL-5 and IL-10 production can be increased (Boonstra et al., 2001), limiting the inflammation and tissue damage associated with excessive Th1 cellular immunity and shifting the balance to a Th2 cell phenotype. 1, 25(OH)$_2$D has also been shown to prevent tissue damage through suppressing the development of Th17 cells and inhibiting the production of inflammatory cytokines by Th17 cells i.e. IL-17 (Chang et al., 2010). Additionally, it has also been shown that treatment of naïve CD4 T cells with 1, 25(OH)$_2$D potently induced the development of regulatory T cells (Treg) which are capable of producing cytokine that block Th1 development (Gorman et al., 2007). Vitamin D also increases synthesis of the primary anti-inflammatory cytokine IL-10 by Treg and dendritic cells (Ginde et al., 2009; Sandhu & Casale, 2010).

In the other major type of adaptive immune cells, B cells, the anti-proliferative effects of 1, 25(OH)$_2$D were initially considered to be indirectly mediated by T helper (Th) cells (Lemire et

al., 1984). However, additional direct effects of 1, 25$(OH)_2$D on B cell homeostasis, including inhibition of memory and plasma cells generation, as well as promotion of apoptosis of immunoglobulin producing B-cells (S. Chen et al., 2007). This control on B cell activation and proliferation may have clinical implications for autoimmune diseases such as B cells producing autoreactive antibodies. Taken together, the expression of the VDR and vitamin D activating enzymes in both T and B cells may play a major role in the pathophysiology of a number of conditions which the immune system is directed at self, i.e. autoimmunity. Indeed, 1, 25$(OH)_2$D administration has prevented and/or treated various experimental models of autoimmune disorders, including; inflammatory arthritis, autoimmune diabetes, experimental allergic encephalitis (a model for multiple sclerosis), and inflammatory bowel disease (Adorini, 2005; Deluca & Cantorna, 2001).

The actions of vitamin D on adaptive immunity appear to be mostly suppressive or inhibitory, and could be considered to paradoxically impair immune responses to pathogens and increase susceptibility to infection. However, recently studies have indicated that vitamin D is crucial in activating and controlling the T-cell antigen receptor and thus enhancing the recognition of antigens by T lymphocytes leading to an activation of the cellular immune response in response to pathogen exposure (Kongsbak, Levring, Geisler, & von Essen, 2013; von Essen et al., 2010). Naïve human T cells have low expression of phospholipase C-gamma 1 (PLC-γ1), a key signalling protein downstream of many extracellular stimuli. This characteristic is associated with low T-cell antigen receptor (TCR) responsiveness in naïve T-cells. However, TCR triggering leads to a large up regulation of PLC-γ1 expression, which correlates with greater TCR responsiveness. Although naïve T-cells do not express the VDR, VDR expression is induced by TCR signalling via the alternative mitogen activated protein kinase p38 pathway. Thus initial TCR signalling via p38 leads to successive induction of VDR and PLC-γ1, which are required for subsequent classical TCR signalling and T-cell activation (von Essen et al., 2010).

These findings indicate that vitamin D is crucial for the activation of the acquired immune system and therefore very important for the effective clearance of viral infection. Therefore, the aforementioned suppressive actions of vitamin D adaptive immunity may be a reaction to prevent exaggerated and excessive inflammatory immune responses following T-cell activation, which potentially cause injury to the host. This is important, as the ideal immune response is rapid, proportionate and effective but finite. Taken together these findings along

with the identification of hundreds of primary 1, 25$(OH)_2$D target genes in immune cells suggests that vitamin D may regulate both innate and adaptive arms of the immune system (**Figure 2.1**).

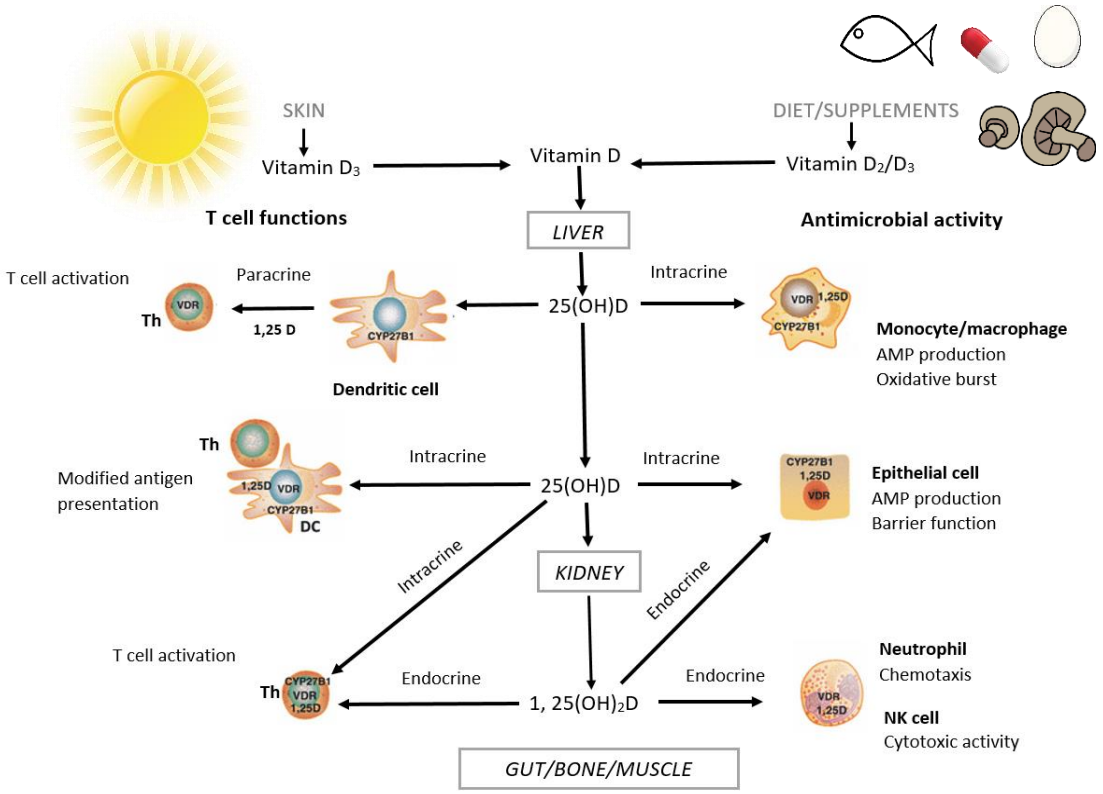

Figure 2.1. Adapted from He et al., 2016 and Prietl et al., 2013. Mechanisms for innate and adaptive immune responses to vitamin D. Ergocalciferol (vitamin D_2) from the diet and cholecalciferol (vitamin D_3) from the diet or produced from the actions of UVB on the skin are metabolised in the liver to form 25 hydroxyvitamin D (25(OH)D) the main circulating form of vitamin D. Target cells such as monocytes, macrophages and dendritic cells expressing the mitochondrial vitamin D activating enzyme 1-α hydroxylase (CYP27B1) and the cytoplasmic vitamin D receptor can the utilise 25(OH)D for intracrine responses via localised conversion to 1, 25-dihydroxy-vitamin D (1, 25$(OH)_2$D; calcitriol also shown as 1, 25 for intracellular locations). In monocytes and macrophages this promotes antimicrobial responses to infection. In dendritic cells, intracrine synthesis of 1, 25$(OH)_2$D inhibits dendritic cell maturation, thereby modulating T-helper (Th) cell function. Th responses to 25(OH)D may also be mediated in a paracrine fashion, via the actions of dendritic cell-generated 1, 25$(OH)_2$D. Intracrine immune effects of 25(OH)D also occur in epithelial cells expressing the VDR and the CYP27B1. However, neutrophils and natural killer (NK) cells do not appear to express CYP27B1 and are likely to be directly affected by circulating levels of 1, 25$(OH)_2$D synthesised by the kidneys or locally produced in and secreted from tissue macrophages and dendritic cells. VDR-expressing Th cells are also potential targets for systemic 1, 25$(OH)_2$D, although intracrine mechanisms have also been proposed. In a similar fashion, epithelial cells can responds in an intracrine fashion to 25(OH)D but may also respond to systemic 1, 25$(OH)_2$D to promote antibacterial responses.

2.5 Using vaccinations to assess *in vivo* immune function

In a bid to explain the role of vitamin D with both innate and adaptive arms of the immune system, the aforementioned studies have relied predominately on *in vitro* assessments of immune function and status. The variety of roles vitamin D orchestrates in innate and adaptive immunity *in vitro*, have been linked to findings of epidemiological studies, which have demonstrated the role of vitamin D in reducing the susceptibility to opportunistic infection (Ginde et al., 2009; Martineau et al., 2017). However, *in vitro* immune assays that involve the extraction of immune cells from their normal environment and analysed in artificial cultures, lack the highly integrated neural and hormonal components within the specific tissue environment in which immune responses usually take place (Akbar et al., 2013). In contrast, as an *in vivo* process, antibody response to vaccination occurs within this dynamic neuroendocrine environment and potentially provides a much better estimation of immune functioning (Burns & Gallagher, 2010). Given the variety of roles vitamin D has in both innate and adaptive arms of the immune system, it is conceivable that vitamin D may also influence the orchestration of immune responses following vaccination.

2. 5.1 Integrated measure of immune function

Clinically, vaccines are a major strategy in combatting infectious diseases. Vaccination with inactivated antigens manipulates the adaptive immune system into producing antibodies that will protect the host against subsequent attacks from the infectious agent. This response is orchestrated without eliciting the symptoms of the disease that would result from inoculation with live pathogens. Antibodies are highly specific protein molecules that bind to invading microorganisms and mark them for destruction or prevent them from infecting cells. When the immune system encounters an antigen for the first time it produces a slower primary immune response primarily orchestrated by the innate immune system at the site of entry. Vaccine antigens are captured, processed and presented by antigen presenting cells (APCs; i.e. macrophages and dendritic cells), which migrate into the lymph nodes where they induce activation and clonal expansion of naïve CD4+ and CD8+ T cells (helper and cytotoxic cells respectively). The subsequent activation and differentiation of naïve B-cells is induced by CD4+ T-cells, which differentiate into memory B-cells and antibody secreting B-cells. These cells, now called memory lymphocytes may persist for many years and function more rapidly upon antigen re-exposure. Firstly, this is explained by more antigen reactive lymphocytes after initial exposure than before exposure. Secondly, the affinity with which B-lymphocytes bind to an antigen is increased, resulting in a more rapid signal transmission for antibody release.

Finally, the previously activated lymphocytes express new proteins on their membranes that promotes more efficient interaction between T and B-lymphocytes. Together, these changes result in a rapid and increased production of antibody. Long term immunity is maintained by memory B and T cells (CD4+ and CD8+) in the blood stream and lymph nodes, as well as in the bone marrow by long lived plasma cells and memory T-lymphocytes (Lang et al., 2013). Therefore, the antibody status follow vaccination represents the culminations of a series of interactions of different immune cells types in various immune compartments occurring within a dynamic neuroendocrine milieu (Burns & Gallagher, 2010). These antibodies can be measured in serum, therefore yielding a quantifiable measures of the final product of the cascade of reactions (Delves & Roitt, 2011).

2.6 Immune modulatory roles of vitamin D in vaccine response

Vitamin D may mediate vaccine response by stimulating APCs, which are pivotal for the initial capturing, processing and presenting of the antigen at the site of vaccination (D'Ambrosio et al., 1998; Penna & Adorini, 2000). In animal models, it has been observed that locally produced 1, 25$(OH)_2$D induced migration of DCs from the site of vaccination to non-draining lymphoid organs, where they can stimulate antigen specific T and B-cells to mount a strong and persistent antibody response to diphtheria vaccination (Enioutina, Bareyan, & Daynes, 2008, 2009). Furthermore, the potential adjuvant effect of vitamin D was observed through co-administration of 1, 25$(OH)_2$D with trivalent influenza vaccine in mice. Both mucosal and systemic specific antibody response were enhanced, and the animals' subsequent ability to neutralize live influenza virus instilled in the nose (Daynes & Araneo, 1994; Daynes, Araneo, Hennebold, Enioutina, & Mu, 1995).

In contrast, the influence of serum vitamin D on the regulation of vaccine induced immunity in humans is far from being clearly demonstrated; with much of the published literature demonstrating disparate findings (table 2.2). Previously, research has predominately investigated the role of vitamin D deficiency in viral respiratory illnesses, and the immune response to trivalent influenza vaccine (TIV) in children and adults. The clinical endpoint to TIV is assessed through hemmagglutination-inhibition (HAI) titre, which is subsequently evaluated for the rate of seroprotection ($\geq$ 4 fold rise in HAI post vaccination) to TIV. Consistently, vitamin D has been shown to have no association with achieving seroprotected or seroconverted status among large cohorts of Canadian children and US adults (Science et al., 2014; Sundaram et al., 2013). Similar findings have also been observed for a variety of

other vaccine antigens (as summarised in table 2.3). Among 85 asplenic patients who received vaccination against *S.pneumoniae* using 7-valent pnemococcal conjugate vaccine (PCV7), MenC and/or haemophilus influenza serotype B. Serum 25(OH)D concentration measured prior to vaccination were similar among both hypo-responders and responders and no association was found between the serum 25(OH)D concentrations and specific antibody titre, assessed 21 days after vaccination, to any single pnemococcal serotype. There was also no association observed between vitamin D concentration and vaccination response to Neissria meningitides type C vaccine. Furthermore, differences could not be assessed in the HiB vaccination group, as there were only two hyporesponders (Peelen et al., 2013).

The lack of association observed between vitamin D and response to vaccination may be due to the variety of vaccinations used in these studies, which vary in terms of the type of antibody they elicit, the relative novelty of the antigen and the typical efficacy of the vaccination in the general population. Furthermore, there are various shortfalls to much of the previously mentioned research examining the link between vitamin D and vaccine induced immunity. The aforementioned cohort studies assessing the influence of vitamin D status on the immune response to TIV conducted among Canadian children and US adults failed to demonstrate an adequate range of vitamin D concentrations. Indeed, Canadian children had vitamin D concentrations exceeding 60 nmol/L (IQR 50-70 nmol/L), whilst US adults had vitamin D concentrations ~80 nmol/L in both year 1 and 2 (Science et al., 2014; Sundaram et al., 2013).

2.6.1 Vitamin D & Hepatitis B vaccine response

The experimental use of the hepatitis B vaccination as a marker of *in vivo* immunity is particularly attractive given that many populations are unlikely to encounter the antigen previously (Burns & Gallagher, 2010). For example, young, healthy adults in the United Kingdom, where the three dose hepatitis B vaccination sequence (0-1-6 months) is only distributed to those in high-risk professions, are largely seronegative at recruitment. The measurement of antibodies (anti-HBs) following the typical three dose series provides an interesting opportunity to examine both primary and secondary responses to the initial and subsequent booster vaccine doses, respectively (Burns & Gallagher, 2010). The latter response tends to be stronger due to quicker recognition of the hepatitis B antigen by memory T-cells formed during the first exposure. It should be acknowledged that there is little variability in response across individuals following either the first vaccination, when only 25% of individuals have protective concentrations of antibody (anti-HBs $\geq$ 10 mIU/mL) (Glaser et al., 1992;

Prather et al., 2012), or the third vaccination, when the majority of recipients have mounted maximal antibody responses (Marsland, Cohen, Rabin, & Manuck, 2006) (**Figure 2.2**). In contrast, there is widespread inter - individual variability in the magnitude of antibody response following the second vaccination (**Figure 2.2**). The range of antibody response following this vaccine dose provides a setting to explore the association between a wide range of vitamin D status and the individual difference in serum antibody response

The strongest support for the relationship between vitamin D and hepatitis B vaccine response has been conducted among chronic kidney disease (CKD) patients. One retrospective study noted a clear association between compromised hepatitis B vaccine seroconversion (defined as post vaccination anti-HBs titre ≥ 10 IU/mL) and vitamin D deficient patients with CKD and very low 25(OH)D status (< 25 nmol/L). This study evaluated antibody response to three doses of 40μg recombinant hepatitis B vaccine among 200 CKD patients (Zitt et al., 2012). Overall, only 57% of patients mounted an antibody response ≥ 10mIU/mL. This blunting in seroconversion was further demonstrated among patients with vitamin D concentrations < 25 nmol/L compared to those who had vitamin D concentrations ≥ 25 nmol/L (45% *vs.* 64%, p = 0.01). Additionally, in a multivariate analysis (logistic regression) model, vitamin D deficiency was one of the independent significant negative predictors of seroconversion. These findings may partially explain why, on average, only 50-60% of end stage renal disease patients seroconvert following hepatitis B vaccination compared to more than 90% of the general population (Buti et al., 1992). However, considering the worldwide prevalence of vitamin D insufficiency in otherwise healthy young adults, investigating the association of vitamin D with hepatitis B vaccine responsiveness in young healthy adults is warranted.

2.6.2 Studying the influence of vitamin D on vaccination response

Studies examining potential roles of vitamin D in immune response to vaccines, as an *in vivo* marker of immunity, should consider several issues. Firstly, studies must encompass all seasons, therefore providing a wide distribution of vitamin D concentrations. Such studies should then be followed up by supplementation studies which seek to boost baseline serum vitamin D concentrations to a threshold important for optimal *in vivo* immune function. However, it is important to highlight that there is no consensus regarding classifications of vitamin D for optimal immune function. Despite this, current EFSA and IOM recommendations advise that avoiding vitamin D insufficiency (25(OH)D < 50 nmol/L) is essential for bone and general health. Also, a comprehensive review that summarises various

studies that have attempted to evaluate threshold concentrations for circulating 25(OH)D in relation to multiple health outcomes concluded that for all endpoints, circulating concentrations of 25(OH)D < 50 nmol/L are associated with adverse effects (Bischoff-Ferrari, 2014). Given that > 50% of otherwise healthy adults are considered to have insufficient vitamin D status during the winter (He, Aw Yong, et al., 2016), examining strategies to avoid vitamin D insufficiency and its potential influence on *in vivo* immune function may be of clinical importance.

As described previously, vaccination induces a multistep immune response initiated by the initial processing of the antigen at the site of vaccination and the eventual development of antigen specific memory T and B cells as effectors of immune protection. Animal research suggests that the critical stages of orchestrating the development of new immunity, termed induction, are most sensitive to immune impairment (Fleshner et al., 1992; Okimura et al., 1986). Limited studies in humans show similar findings. Where stress in close proximity to the first immune challenge has an immune modulatory effect (Harper Smith et al., 2011), but stress close to the recall has a greatly reduced (Harper Smith et al., 2011) or no effect (Smith et al., 2004). Whether vitamin D is important for initial capturing and processing of an antigen at the site of vaccination or the subsequent downstream effects leading to the development of immune memory is not well characterized. However, limited evidence does indicate higher vitamin D status at the time of initial vaccination may have a greater modulating effect on subsequent *in vivo* antibody response. Indeed, it has been shown that individuals who received vitamin D for 9 weeks followed by tetanus toxoid booster immunization displayed higher IgG responses compared to those receiving a placebo (Heine et al., 2011). Comparatively, there are no studies assessing whether changing vitamin D status following vaccination influences the subsequent development of *in vivo* antibody response. In order to investigate this, experimenters would need to ensure that participants display low vitamin D status at the time of initial vaccination. This might be achieved by commencing a study during the winter, when vitamin D is at its typical seasonal nadir. Following vaccination, investigators should seek to increase vitamin D status. Two main methods which can be adopted to increase vitamin D concentrations, are firstly; taking a daily oral vitamin D supplement during the and secondly, where possible, practicing safe summer sunlight exposure either naturally or via artificial exposure to UVR. (He, Aw Yong, et al., 2016).

Table 2.2. Summary of studies evaluating the influence of vitamin D on vaccine immunogenicity in humans.

Reference	Vaccine & dose	Study		VD Suppl.		VD Status (nmol/L)	Outcome measure	Results
		Design	Population	Dosage	Duration	Baseline		
Principi et al., 2013	Influenza	RCT	N = 116 children (53% male, age 3 ± 1 years)	1,000 IU/day *vs.* placebo	4 months	NA	HI antibodies to influenza strains	NS
Science et al ., 2014	Influenza	CS	N = 221 children [48% male, age 9 (3-15 years)]	NA		61 (51-72)[a]	HI antibodies to influenza strains	NS
Sundaram et al., 2013	Influenza	CS	N = 1103 healthy adults Age > 50 years, 2008 - 09 (39% male, n=591), 2009 – 10 (38% male, n = 509)	NA		2008-09: 78±28[b] 2009-10: 78±25[b]	21-28 day post HI antibodies to influenza strains	NS
Chadha et al., 2011	Influenza	CS	N = 35 Prostate cancer patients [age 68 (53 – 79 years)]	NA		45 (9-72)[a]	HI antibodies to influenza strains	Baseline VD (continuous and categorical) related to serological response

Reference	Vaccine & dose	Study		VD Suppl.		VD Status (nmol/L)	Outcome measure	Results
		Design	Population	Dosage	Duration			
Crum-Cianflone et al., 2016	Influenza	CS	N = 128 (n = 64 HIV infected *vs.* n = 64 HIV uninfected adults) (age 18 – 50 years)	NA		HIV: 72±43[b] U-HIV: 70±42[b]	HI antibodies to influenza strains	NS
Heine et al., 2011	Tetanus toxoid	CS	N = 32 Age not given	2000 IU/day (oil)	10 weeks	NA	The TT-specific IgG	The TT-specific IgG boost efficiency was marginally higher in the vitamin D group (P = 0.04).
Zitt et al., 2012	Hepatitis B (HBVAXPRO + Engerix B, 40 µg	CS	N = 200 CKD patients (75% male, age 64 ± 15 years)	NA		NA	CIA; Anti-HBs	< 25 nmol/L had lower seroconversion than > 25 nmol/L. Deficiency independent predictor (OR 0.496; P = 0.038)

Reference	Vaccine & dose	Study		VD Suppl.		VD Status	Outcome measure	Results
		Design	Population	Dosage	Duration			
Zheng et al., 2014	Bacille Calmette-Guerin (BCG) vaccine	RCT	N = 629 Revaccinated Chinese infants (3, 6 and 12 months old)	Combined Vitamin A (1500 IU/day)+ Vitamin D (500 IU/day)	3 months	NA	BCG scars and PPD induration	Rate of positive PPD responses higher in the supplementation compared to control group (96.1% *vs.* 89.7%)

CS: Cross sectional; RCT: Randomised control trial; VD: vitamin D; NA: Not applicable; NS = no significant difference.

[a.] Data are Mean ± SD

[b.] Data are Median (interquartile range)

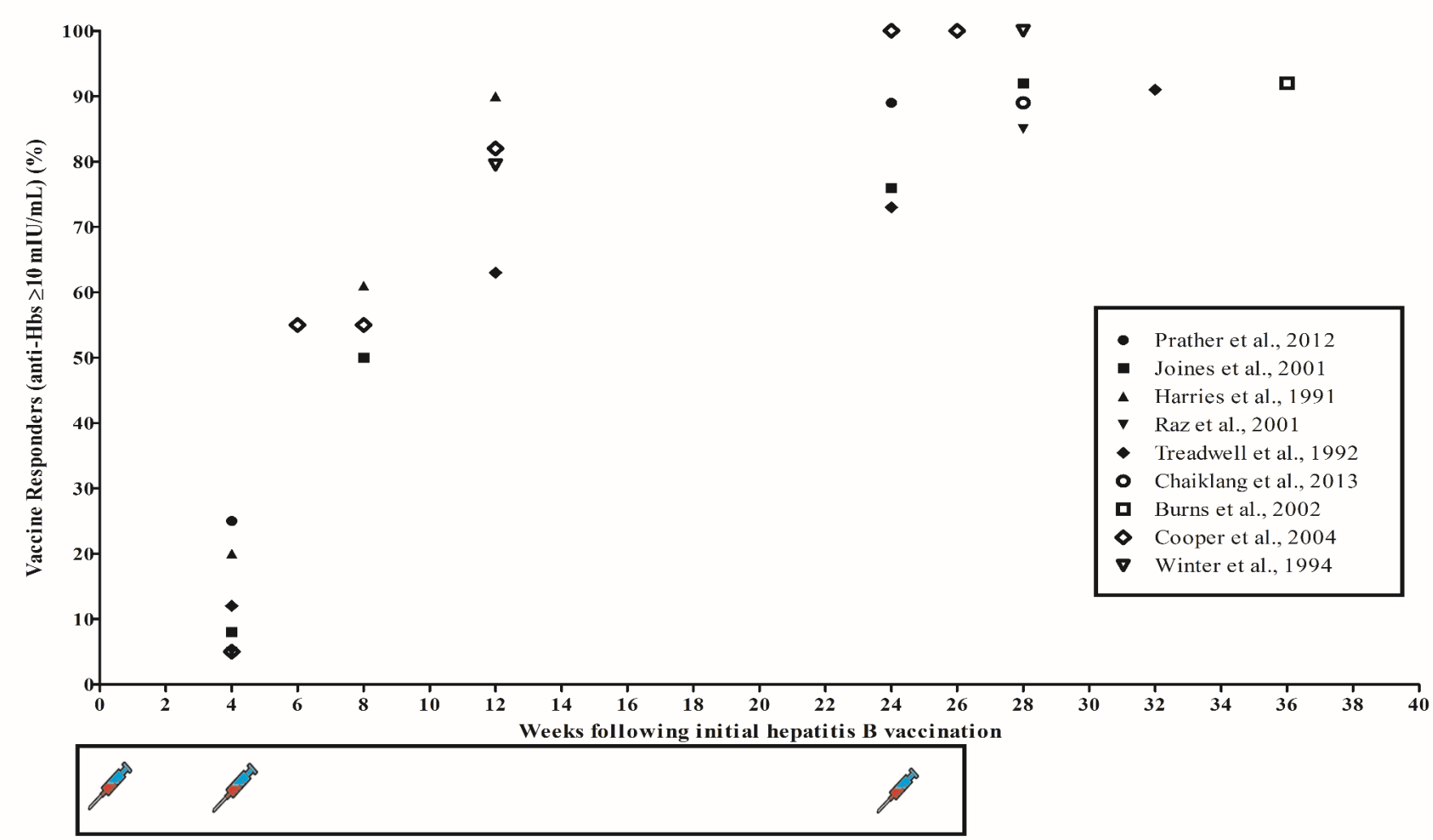

Figure 2.2. Typical anti-HB percentage response following each hepatitis B vaccine dose (20µg recombinant hepatitis B vaccine).

2.7 Vitamin D supplementation strategies

To date no studies have directly examined the influence of increasing vitamin D status on *in vivo* immune measures i.e. antibody response following vaccination. The majority of research conducted has sought to optimise vitamin D status and defence against URI by adopting the strategy of supplementing individuals with oral vitamin D_3 supplements. These studies have been compiled and reviewed in a recent meta-analysis (Martineau et al., 2017). However less understood, and to date not researched, is the potential of practicing safe summer sunlight exposure to increase vitamin D status in an attempt to enhance defence against immune suppression.

2.7.1. Oral vitamin D supplementation on vitamin D status, and vaccination response

Although vitamin D_2 and D_3 are available as oral supplements, vitamin D_3 supplementation is more commonly used as it has a greater efficacy in raising serum 25(OH)D compared to vitamin D_2 (Houghton & Vieth, 2006). Current evidence indicates that orally administered supplemental vitamin D (D_2 & D_3) can enhance innate immune response to mycobacterial infection (mycobacterium bovis in the BCG-lux assay) (Martineau et al., 2007) and increases circulating concentration of cathelicidin (Bhan et al., 2011). However, few studies have investigated the influence of increasing vitamin D concentration and subsequent antibody response following vaccination. Two studies have examined the influence of vitamin D supplementation. Firstly, a placebo blinded trial involving an intramuscular administration of 1.0 μg 1, $25(OH)_2D$ at the site adjacent to influenza vaccination did not find any significant differences in post vaccination HAI response between the treatment and placebo groups (Kriesel & Spruance, 1999). The second study was conducted among a cohort of 116 children (mean age; 3 ± 1 y) who had not previously received influenza vaccination. Fifty-nine children commenced 4 months of vitamin D supplementation (1,000 IU/day) at the same time as the first dose of TIV. A second dose of TIV was administered ~ 1 month later, and a blood sample was drawn at the end of the 4 month supplementation period. Baseline vitamin D concentrations were similar between the groups at the start of the study; however, as before, this study revealed no significant difference in post vaccination immune response between the treatment and placebo groups (Principi et al., 2013).

Additionally, in the RCT trial conducted by Kriesel and Spruance (1999), baseline and post calcitriol serum vitamin D status was not measured. Further, significant pre-vaccination HAI titre were measured in all participants; indicating that participants had considerable immunity

to the three influenza vaccine strains. Since a clear inverse relationship exists between pre-immunization serum HAI titres and antibody response after vaccination, this could have masked potential vaccine enhancing effects of 1, $25(OH)_2D$. A mixture of primary, secondary and tertiary responses can be yielded when including repeat antigens such as the influenza virus.

However boosting from a higher starting concentration of serum 25(OH)D may provide no additional benefit (Bryson, Nash, & Norval, 2014). This suggestion has been recently supported in the aforementioned meta-analysis, which showed that the protective effects of vitamin D were strongest in those with profound vitamin D deficiency at baseline (Martineau et al., 2017). Another possible explanation could be the variable dosing regimens adopted by the studies. Indeed, it has been shown that daily or weekly supplementation without additional bolus are protective against acute respiratory tract infections, whereas regimens containing large and less frequent boluses as adopted by Murdoch and colleagues do not (Martineau et al., 2017). Therefore, randomised control trials of frequent oral vitamin D supplementation are sorely needed in young healthy individuals around the winter time nadir in serum 25(OH)D.

To a lesser extent, the role of vitamin D has been explored in the orchestration of immune response to other vaccine challenges. For instance, a higher rate of purified protein derivative (PPD) response, following routine BCG vaccination, has been observed among a cohort of infants co-supplemented with vitamin A and D (500 IU/day) for 3 months compared to a control group (Zheng et al., 2014). Additionally, tetanus toxoid specific IgG booster efficacy has been shown to be higher among 20 healthy adults who received oral doses of vitamin D_3 supplementation for a period of 9 weeks (2,000 IU/day) compared to those who received an oral placebo (Heine et al., 2011).

2.7.2 UVB radiation on vitamin D status and vaccination response

For a range of skin colours and latitudes between 30-60 °N, the majority of vitamin D can be obtained through short lasting skin exposures to natural UVB irradiation through summer sunlight. To date, however, it remains unknown if UVB exposure of the skin has benefits on immune function. It could be speculated that UVB exposure (either from sunlight or a sun cabinet) could have a positive influence on immune function independent of the synthesis of vitamin D. For example, UVR generates nitric oxide locally at the skin which has been associated with benefits to cardiovascular health via a decrease in systemic blood pressure

(Juzeniene & Moan, 2012). As well as its effects on vasodilation, nitric oxide may influence neurotransmission, immune defence, regulation of cell death (apoptosis) and cell motility (Juzeniene & Moan, 2012). The potential for mood enhancement (possibly mediated via increased β-endorphins) and stress reduction with skin sunlight or artificial UVB exposure to influence immune function should not be overlooked. Indeed, it is possible, but remains unknown, that UVB exposure of the skin improves immune function greater than the equivalent oral vitamin D_3 supplementation due to enhanced concentrations of nitric oxide, mood or some yet unknown mechanism.

UVR has been shown to diminish resistance to certain local and systemic infection in experimental animal models (Enioutina, Visic, & Daynes, 2002). However, the evidence in humans is much more limited. Only one study has attempted to study how an intensive period (5 days) of UV treatment prior to vaccination may influence subsequent vaccine response (Sleijffers et al., 2001). In this instance despite observing a modulating influence of CHS response to the novel antigen DPCP, no influence was observed on the antibody response to hepatitis B vaccine. However, it should be noted that the investigators were trying to investigate potential immunosuppression because of more 'extreme' personal UV doses. In this instance the authors included the use of fluorescent lamps with UV emission removed from that of sunlight, specifically exposure to personalized (MED-related) UV doses rather than a standardized challenge, and near total-body surface exposure, which is unlikely to occur in everyday life. A method to replicate safe summer sunlight exposure using sub-erythemal solar simulated radiation in a laboratory based irradiation cabinet has been developed by Rhodes and colleagues (Rhodes et al., 2010). During the winter months, when vitamin D status was low, this method restored adequate circulating concentrations of vitamin D (25(OH)D level > 50 nmol/L) in the majority of volunteers. Importantly, in line with policy recommendations in the UK, this method simulates summer sunlight exposure (at latitudes between 30-60 °N for most skin types), on several occasions each week, for ~15 minutes wearing t-shirt and shorts without producing sunburn (Webb, Kift, Berry, & Rhodes, 2011). Promising work shows a threefold increase in circulating Tregs that correlated positively with the change in serum 25(OH)D in patients with psoriasis after 4 weeks of photothrepahy (Milliken et al., 2012). However, whether the method of simulating summer sunlight exposure to restore adequate circulating vitamin D in the winter improves immunity and host defence remains largely unknown and requires investigation.

2.8 Thesis objectives

With this information in mind, the objectives of this thesis were to investigate; 1, the influence of vitamin D status before initial vaccination on secondary anti-HBs response; 2, the efficacy of increasing and maintaining 25(OH)D with an oral vitamin D_3 supplement compared to simulated safe sunlight exposure; 3, the influence of increasing vitamin D status by safe and government recommended simulated sunlight exposure and matched oral vitamin D_3 doses after the initial vaccination on secondary anti-HBs response.

CHAPTER THREE

General Methods

3.1 Ethical approval. Studies received ethics approval from the UK Ministry of Defence Research Ethics Committee (**Chapters 4 & 6**) or from the local Ethics Committee (School of Sport, Health and Exercise Sciences, Bangor University, **Chapter 5**) and were conducted in accordance with the declaration of Helsinki (2013). The nature and purpose of each study was fully explained in writing and verbally before participants provided written informed consent.

3.2 Participant characteristics. Participants were recruited from a representative sample of British men starting the Combat Infantryman's Course at the Infantry Training Centre, Catterick (Latitude 54°N) (**Chapter 4 & 6**) and women entering the Common Military Syllabus for recruits at The Army Training Centre, Pirbright (Latitude 51°N) respectively from June 2014 to November 2015 (**Chapter 4**), or recreationally active students 18-33 years of age who had no current injury or illness (**Chapter 5**). Participants in **Chapters 5 and 6** experienced no prolonged sun exposure in the three months prior to testing or were currently using sunbeds or taking multivitamins containing vitamin D, vitamin D supplements, fish oils or photoactive medication. Additionally, all participants had a sun reactive skin type I to III (Fitzpatrick, 1988), had no previous history of skin cancer, photosensitivity or systemic lupus erythematosus. Furthermore, participants in **Chapters 4 and 6** had no previous history of exposure to the hepatitis B vaccine.

3.3 Anthropometry. Height and body mass were measured in light clothing with shoes removed, using a stadiometer and digital platform scale (SECA 703, Birmingham, UK), during initial medical assessment at both army training centres (**Chapters 4 & 6**), or during an initial baseline visit at the School of Sport, Health & Exercise Sciences (Bangor University) (**Chapter 5**). These measurements were then used to calculate body mass index (BMI; kg/m^2).

3.4 Lifestyle questionnaire. Alcohol and smoking use were determined from participants responses to a lifestyle questionnaire, which was tested by Army Recruitment and Training Division for comprehension and repeatability, with a test-retest intraclass correlation coefficients > 0.76 and percentage agreement > 93%, for the aforementioned questions. On the questionnaire participants also recorded their previous night's sleep and morning awake times,

for the determination of sleep duration, and rated their previous night's sleep quality from 1 = very poor to 4 = very good (Prather et al., 2012).

3.5 Hepatitis B vaccination. During initial medical assessments, Army medical personnel administered the first 20-µg dose of recombinant hepatitis B vaccine (Engerix-B, Smithkline Beecham Pharmaceuticals, Uxbridge, UK) into the deltoid muscle (**Chapters 4 & 6**). Army medical personnel administered a second 20-µg hepatitis B vaccine dose 1 month after the first dose.

3.6 Blood (Chapters 4, 5 & 6). Whole blood samples were collected by venepuncture from an antecubital vein into two plain vacutainer tubes (Becton Dickinson, Oxford, UK) and left to clot for one hour at room temperature. Blood samples were centrifuged at 1500g for 10 minutes in a refrigerated centrifuge (4°C). Serum was then aliquoted into universal and eppendorf tubes and immediately frozen and stored at –80°C for later analysis.

3.6.1 Analysis of vitamin D metabolites. All serum analyses for vitamin D metabolites were determined in a Vitamin D External Quality Assurance Scheme accredited laboratory, with the LC-MS/MS method of analysis, which is regarded as the gold standard for assessment of vitamin D metabolites (Fraser & Milan, 2013). Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of $25(OH)D_3$, $25(OH)D_2$ (**Chapter 4, 5 & 6**) and 24, $25(OH)_2D_3$ 24, $25(OH)_2D_2$ (**Chapter 6**) was performed using a Micromass Quattro Ultima Pt mass spectrometer (Walters Corp., Milford, Ma, USA). Measurements of total 1, $25(OH)_2D$ were carried out using a commercially available enzyme immunoassay kit (IDS, Boldon, UK, **Chapter 4 & 6**). Duplicate samples underwent immunoextraction with a 1, $25(OH)_2D$ specific solid phase monoclonal antibody and incubated overnight with sheep anti - 1, 25 $(OH)_2D$. 1, $25(OH)_2D$ linked biotin was added the next day, followed by horseradish peroxidase labelled avidin to selectively bind to biotin complex. After a wash step, colour was developed using a chromogenic substrate (TMB). The absorbance of the stopped reaction mixtures were read in a micro titre plate spectrophotometer (Multiskan Go, Thermo Scientific, Finland) at a wavelength of 450 nm.

3.6.2 Secondary anti-HBs titre response. Baseline and secondary serum anti-HBs titres were determined quantitatively using an anti-HBs enzyme-linked immunoassay kit (DiaSorin,

Saluggia, Italy, **Chapters 4 & 6**) according to the manufacturer's instructions. A result of ≥ 20mIU/mL indicated that a sample was 'Positive' for anti-HBs, indicative of anti-HBs titre concentrations consistent with protective immunity against HBV infection and therefore not re-tested. A sample below < 0.1 mIU/mL indicated that the result was below the test's limit of detection (LoD). Therefore, the sample was considered 'Negative' for anti-HBs and the individual was not immune to HBV infection. In such cases, the samples were retested in duplicate in order to confirm the initial result. Additionally, results of > 0.1 mIU/mL and < 20 mIU/mL indicated that sample was 'indeterminate' for anti-HBs and was tested in duplicate. If both repeats were > 10mIU/mL, the specimen was considered positive for anti-HBs. If the quality control results fell outside the established acceptable range (8.0 - 17.7mIU/mL) on specific assay plates, a sample of participant specimens from the plate, which fell within the 95% confidence interval around 10 mIU/mL and 3 standard deviations around 100 mIU/mL were repeated. A summary of the rationale to accept or re run samples is provided by the ELISA algorithm (**Appendix A**). If antibody titres were ≥ 1,000 mIU/mL, the highest detectable by the enzyme linked immunoassay methods, samples were diluted in a serial dilution with negative control.

To determine inter-assay variability an external assayed human quality control was aliquoted in triplicate across all analyzed ELISA plates (VIROTROL II-A, BioRad). The intra-assay coefficient of variation and inter-assay variability of the human quality control samples are summarised in **Appendix B**.

3.7 Vitamin D supplementation (Chapter 5 & 6). Both intervention strategies (SSR and ORAL) aimed to achieve and maintain serum 25(OH)D at ≥ 50 nmol/L as recommended by EFSA and the IOM (European Food Safety Authority, 2016; Institute of Medicine, 2011). Participants completed a 4-week restoration phase, necessary because 25(OH)D was it its winter nadir at week 1 followed by a 8 - week maintenance phase.

3.7.1 Oral vitamin D_3 supplementation. Those assigned to oral vitamin D_3 supplementation (ORAL) consumed one 1000 IU vitamin D_3 capsule each day for 4 weeks (restoration phase) and then 400 IU vitamin D_3 capsule once daily for 8 weeks (maintenance phase) (PureEncapsulations, Sudbury, MA, USA). Those assigned to oral placebo (ORAL-P) were given inactive capsules of identical size and appearance to the vitamin D_3 capsule once daily

for 12 weeks. Independent analysis (by NSF International Laboratories, Ann Arbor, Michigan, USA) confirmed oral supplement vitamin D_3 content to be 1090 IU, 460 IU and 0 IU in the 1000 IU, 400 IU and placebo labelled capsules respectively.

3.7.2 Solar simulated radiation (SSR). Those assigned to SSR received UV radiation using a whole body irradiation cabinet (Hapro Jade, Kapelle, The Netherlands) three times per week for 4 weeks and then once per week for 8 weeks. At each visit, wearing protective goggles, with standardised T-shirts and shorts to expose approximately 35% (**Chapter 5**) and 40% (**Chapter 6**) skin surface area. Participants received a 1.3 standard erythemal dose (SED) by being exposed to UV radiation for approximately 3 minutes. This dose is equivalent to 15 minutes midday summer sun exposure to each of the ventral and dorsal surfaces of the body in northern England (Rhodes et al., 2010). The cabinets were fitted with Arimed B (Cosmedico, Stuttgart, Germany) fluorescent lamps with a UV radiation emission spectrum similar to sunlight (290-400 nm, 95% UVA: 320-400 nm, 5% UVB: 290-320 nm), which were calibrated and monitored with a radiometer. Those assigned to placebo SSR were treated identically to those assigned to SSR except that the irradiation cabinet was fitted with a novel UV blocking film (**Chapter 5**) or a polycarbonate plastic, which blocked the transmission of UVR to the participants (**Chapter 6**).

Participant compliance to interventions were determined by experimenter counts of SSR and SSR-P exposures; and by counting unused capsules returned each week. Intervention compliance was documented, and only participants who achieved at least 80% compliance for both the restoration and the maintenance phases were included in the final analysis, as described previously (Macdonald et al., 2013).

3. 8 Statistical analysis. All statistical analyses were completed using SPSS Statistics 22 (IBM, Armonk, New York, USA). Due to non-normal distribution of secondary anti-HBs titre response, the variable was categorised as 'non-responders' (anti–HBs <10 mIU/mL) and 'responders' (anti-HBs ≥ 10mIU/mL) (Huzly, Schenk, Jilg, & Neumann-Haefelin, 2008) **(Chapter 5 and 6)**. Data in the text and tables are presented as mean (SD), unless otherwise stated, and statistical significance was accepted at $P < 0.05$. Data were checked for normality and sphericity. All statistical analysis was conducted using SPSS software.

CHAPTER FOUR

The influence of vitamin D on secondary hepatitis B antibody response

4. 1 Summary

Vitamin D insufficiency (serum 25(OH)D < 50 nmol/L) is widespread yet the effect of vitamin D on the development of *in vivo* immunity, such as secondary antibody response to hepatitis B vaccination (anti-HBs), in young healthy adults is unknown. Therefore the current study aimed to investigate the influence of vitamin D status on the development of secondary anti-HBs response in a prospective cohort study. Four hundred and forty seven (272 males, 175 females) recruits aged 22 ± 3 years were enrolled upon entering the British Army from June 2014 to November 2015. Participants received two doses of the hepatitis B vaccine, separated by 4 weeks. Blood samples were collected immediately after the first 20-μg dose of recombinant hepatitis B vaccine and analysed for vitamin D status (25(OH)D and 1, 25(OH_2D) and hepatitis B antibody titres (anti-HBs). A second blood sample was collected 13 weeks later, ~8 weeks after the second vaccination dose to assess secondary anti-HBs titres. Serum 25(OH)D and 1,25$(OH)_2$D were lower and secondary hepatitis B vaccination responses were poorer in winter than in summer (winter *vs.* summer; serum 25(OH)D [mean ± SD, men; 36 ± 16 *vs.* 80 ± 32 nmol/L, women; 43 ± 20 *vs.* 95 ± 29 nmol/L], serum 1,25$(OH)_2$D [mean ± SD, men; 116 ± 28 *vs.* 136 ± 35 pmol/L, women; 139 ± 36 *vs.* 178 ± 57 nmol/L], secondary anti-HBs ≥ 10 mIU/mL [%, men; 41 *vs.* 58, women; 50 *vs.* 77]). Vitamin D insufficiency was overall more prevalent among men than women (51% vs. 31%, P ≤0.01) and men who presented with vitamin D insufficiency (serum 25(OH)D <50 nmol/L) at the time of initial hepatitis B vaccination mounted a poorer secondary anti-HBs ≥ 10 mIU/mL compared to those who were vitamin D sufficient (anti-HBs ≥10 mIU/mL; OR 1.6; 95% CI 1 – 2.6; P <0.05). Both the winter season and vitamin D insufficiency, in men, at the time of initial vaccination was associated with poorer hepatitis B vaccination in a prospective cohort study. These data indicate that vitamin D may be an important modifiable factor the development of *in vivo* immunity, particularly in men.

4. 2 INTRODUCTION

Prophylactic vaccination is a cornerstone of the public health strategy to prevent disease such as hepatitis B (Schuchat, 2011), which affects ~ 350 million people and kills a further ~ 686,000 per year worldwide due to related liver disease (Mortality & Causes of Death, 2015). Prophylactic vaccination is particularly important for those persons at increased risk such as health care professionals and patients regularly exposed to bodily fluids (Public Health England, 2017; Schillie et al., 2018). The effectiveness of the standard hepatitis B vaccination course varies with 10-15% of adults responding inadequately by producing too few antibodies, as assessed by anti-hepatitis B surface antigen immunoglobulin G response status < 10 mIU/mL following hepatitis B vaccination (anti-HBs titres < 10 mIU/mL) (Public Health England, 2017). Factors considered to contribute to an inadequate hepatitis B vaccine response include male sex, age > 40 years, BMI ≥ 25, smoking, poor sleep and psychological stress (Averhoff et al., 1998; Glaser et al., 1992; Prather et al., 2012; Roome, Walsh, Cartter, & Hadler, 1993; Westmoreland, Player, Heap, & Hammond, 1990; Wood, MacDonald, et al., 1993; Yang et al., 2016).

Vitamin D may also have a modulating role in hepatitis B vaccine immunogenicity. Discovery of the vitamin D receptor (VDR) in almost all immune cells, and the variety of roles vitamin D has in both the innate and adaptive arms of immunity *in vitro* (Chang et al., 2010; He, Handzlik, et al., 2013; Liu et al., 2006; Wang et al., 2004), imply the importance of vitamin D in the upregulation of immune response (Baeke et al., 2010). Vitamin D may mediate vaccine response through its interaction with antigen presentation (Lemire, 1995), dendritic cell migration and the subsequent activation of T and B cell antibody response (Enioutina et al., 2008, 2009; von Essen et al., 2010). To date the association between vitamin D status (serum 25(OH)D) and hepatitis B vaccination response in humans is unclear as previous investigations have studied persons with comorbidity and report conflicting findings (Jhorawat et al., 2016; Zitt et al., 2012). Whether vitamin D status or supplementation influences hepatitis B vaccination in healthy adults is unknown. An understanding of modulating roles of vitamin D in vaccine response is of importance, given that more than 50% of young healthy adults fail to meet Institute of Medicine (IOM) and European Food Safety Authority recommended vitamin D sufficiency (serum 25(OH)D concentration ≥ 50 nmol/L; (European Food Safety Authority, 2016; Institute of Medicine, 2011)) during winter months (He, Aw Yong, et al., 2016).

The current study aimed to investigate the influence of vitamin D status on the development of *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccine

(anti-HBs) among a large population of healthy young men and women. This model of *in vivo* immunity assessed antibody response following the second vaccination of the typical three dose hepatitis B vaccine sequence, and was selected based on our volunteers being naïve to the hepatitis B vaccine antigen, and the widespread inter-individual variability in the magnitude of antibody response at this time (Szmuness et al., 1980). We examined changes in vitamin D metabolites (25(OH)D and 1, 25$(OH)_2$D) and anti-HBs response status across the seasons. It was hypothesised that more participants would mount secondary anti-HBs response (≥ 10 mIU/mL) if they were vaccinated during the summer, when vitamin D concentrations are typically higher, in comparison to winter when they are lower.

4. 3 METHODS

Participant recruitment and exclusion criteria. One thousand and twelve participants were enrolled into the prospective cohort study upon entering the British Army between June 2014 and November 2015. Men were recruited from the Infantry Training centre, Catterick, UK (latitude 54°N), and women were recruited from the Army training centre, Pirbright, UK (latitude 51°N). Eligible participants were ≥ 18 years of age and cleared Army initial medical assessment. Participants were excluded from statistical analysis if their medical records documented previous exposure to hepatitis B vaccination; or if this was later confirmed by measureable antibody titres against hepatitis B surface antigen (anti-HBs) detected in week 1 samples (anti-HBs > 0 mIU/mL). Participants were also excluded if vaccination schedules were atypical, defined as; the first two vaccine doses not being administered within 4-weeks of each other, or the collection of blood to assess the secondary hepatitis B vaccine response occurring < 4-weeks after the second vaccine dose.

Experimental design and procedures. Before participants commenced 13-weeks of Army Phase One basic training, they completed an initial medical assessment. During the initial medical assessment participants received their first 20-μg dose of recombinant hepatitis B vaccine into the deltoid muscle (Engerix–B, Smithkline Beecham Pharmaceuticals, Uxbridge, UK) (**Chapter 3**, **Figure 3.1**). After vaccination, a venous blood sample was collected for the determination of anti-HBs titre, serum 25(OH)D and 1, 25$(OH)_2$D concentrations. At the initial medical assessment we also collected baseline measurements of: height and body mass in light clothing with shoes removed by stadiometer and digital platform scale (SECA 703, Birmingham, UK); sex, ethnicity, alcohol, smoking, sleep and mood by questionnaires. Self-

reported consumption of alcohol and smoking use was obtained from a lifestyle questionnaire that had good comprehension and readability (test-retest intra-class correlation coefficients > 0.76 and percentage agreement > 93%). Before receiving their initial hepatitis B vaccination participants also completed a shortened (24 item) Brunel mood scale (BRUMS) (Terry, Lane, Lane, & Keohane, 1999). This contained a series of descriptive words/statements, and subjects self-reported how these best these words/statement reflected their mood both at the that present time and during the week proceeding on each of these areas using a 5-point Likert scale (0 = 'not at all,' 1 = extremely). The BRUMS scale was also to include questions assessing sleep duration and quality, described and used previously by Prather et al (Prather et al., 2012). Following their model, participants in our study were asked to report the time they went to sleep and awoke the night before vaccination. Sleep duration was calculated as the number of hours and minutes elapsed between the time they reported going to sleep and the time they reported waking. Participants also rated the previous night's sleep quality from 1 = very poor to 4 = very good. In line with the typical hepatitis B vaccination schedule, participants received a second 20-μg hepatitis B vaccine dose one month after the first. A second venous blood sample was collected 8 ± 1 week after the second hepatitis B vaccine dose (3-months after the first hepatitis B vaccine dose) for the determination of secondary serum anti-HBs, the primary outcome measure. Incidence of physician-diagnosed upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI) and gastrointestinal infections were retrieved from the participant's Army medical records for the 13-week period of training.

Biochemical analyses. For blood sample preparation, storage and analyses refer to **Chapter 3, section 3.6**. Intra and inter assay CVs for the assessment of anti-HBs using ELISA (DiaSorin, Saluggia, Italy) were 4.9% and 10.2% respectively (**Appendix B**). Serum 25(OH)D was measured with high-pressure liquid chromatography tandem mass spectrometry; and serum 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) using the DiaSorin LIAISON XL $1,25(OH)_2D$ chemiluminescent immunoassay (Stillwater, Minnesota, USA) method. Analyses were performed in a Vitamin D External Quality Assurance Scheme certified laboratory (Bioanalytical Facility, University of East Anglia, Norwich, UK).

Statistical analysis. We estimated a minimum sample size of 167 was required, using the anticipated difference in hepatitis B vaccine responder rate of 20% between individuals displaying low *vs.* high vitamin D status as previously observed by Zitt et al (Zitt et al., 2012) with a type 1 error (two-tailed) of 5% and a power of 80%. All analyses were conducted with

data for men and women combined and separated, due to the established differences in antibody response following hepatitis B vaccination between men and women (Rendi-Wagner et al., 2001; Westmoreland et al., 1990; Wood, MacDonald, et al., 1993). Primary analysis were chi-square analyses, comparing the percentage of hepatitis B vaccine responders with sufficient (serum 25(OH)D ≥ 50 nmol/L) and insufficient (serum 25(OH)D < 50 nmol/L) vitamin D status. This analysis was followed up with sex specific logistic regression models to determine the influence of vitamin D sufficiency (serum 25(OH)D < or ≥ 50 nmol/L) on secondary hepatitis B vaccination response. For logistic regression analyses, odds ratios (OR) and 95% confidence intervals were included as an estimate of relative risk. In addition, Kruskal-Wallis tests were conducted to compare the percentage of hepatitis B vaccine responders across 1, $25(OH)_2D$ terciles and followed up with post-hoc Mann-Whitney *U*-tests. One-way ANOVA and Kruskal-Wallis tests were conducted to compare serum vitamin D (25(OH)D, and $1,25(OH)_2D$), percentage of participants displaying sufficient vitamin D status (serum 25(OH)D ≥ 50 nmol/L) and the percentage of hepatitis B vaccine responders across season. Where significant differences in serum 25(OH)D and the percentage of participants displaying vitamin D sufficiency were observed, post-hoc pairwise comparisons were conducted using Bonferroni adjusted *t*-tests and Mann-Whitney *U*-tests, respectively. One-way ANOVA and Kruskal-Wallis tests were also used to analyse seasonal variation in baseline participant demographics, anthropometrics, lifestyle characteristics and 'all cause illness' data (URTI, LRTI and gastrointestinal infection) retrieved from medical records. Subsequent Bonferroni adjusted t-tests and Mann-Whitney *U*-tests were used to assess differences in baseline participant demographics, anthropometrics, and lifestyle characteristics between men and women within the seasons. Irrespective of season, demographic, anthropometry, vitamin D (serum 25(OH)D & $125(OH)_2D$), percentage of secondary hepatitis B vaccine responders and lifestyle behaviors were compared between men and women by independent samples *t*-test and Mann-Whitney *U*-tests for normally and non-normally distributed data, respectively. Serum 25(OH)D across the IoM classifications were compared between men and women using chi-square analysis.

4. 4 RESULTS

Participants flow, characteristics and serum 25(OH)D IOM classifications. A total of 1012 British male and female military recruits who were ≥ 18 years and originally volunteered for the study from June 2014 to November 2015. Participants were enrolled throughout the year,

20% in winter (December-February), 14% in spring (March-May), 26% in summer (June-August), and 40% in autumn (September-November). Participant flow, drop out and exclusion prior to biochemical and statistical analysis is summarized **Figure 4.1.** The baseline demographics, anthropometrics, lifestyle behaviours, vitamin D metabolites and secondary hepatitis B vaccine response for the 447 participants included in the final analyses are summarised in **Table 1** (272 men, 175 women). Of note, vitamin D deficiency (serum 25(OH)D < 30 nmol/L) and insufficiency (serum 25(OH)D < 50 nmol/L) was more prevalent in men than women (men *vs.* women, < 30 nmol/L; 21% *vs.* 9%, < 50 nmol/L; 29% *vs.* 22%; $P < 0.05$, **Figure 4.2).** Overall, 55% of participants responded to the hepatitis B vaccination. However, more women were secondary hepatitis B vaccination responders compared to men (anti-HBs ≥ 10 mIU/mL; 65% *vs.* 49%, Table 1**).**

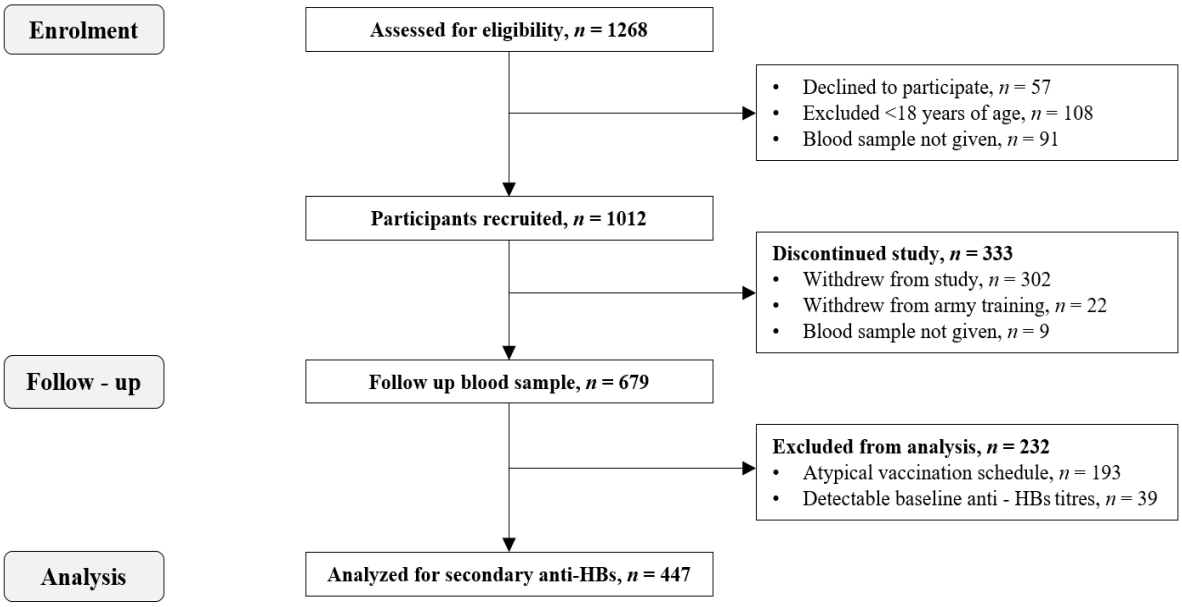

Figure 4.1. Flow diagram indicating the numbers of participants assessed for eligibility, recruited, available at follow-up and analysed. Anti-HBs; antibodies against hepatitis B antigen.

Seasonal variation in vitamin D (serum 25(OH)D and 1, $25(OH)_2D$) and secondary hepatitis B vaccine response. Baseline winter serum 25(OH)D was lower than summer and autumn in men; and lower than spring, summer and autumn in women ($P < 0.05$; **Figure 4.3A**).

During winter, only 15% of men and 26% of women were vitamin D sufficient (baseline 25(OH)D < 50 nmol/L; **Figure 4.3B**). There were similar seasonal variations in serum 1,25$(OH)_2$D, with winter serum 1, 25$(OH)_2$D lower than summer and autumn in men (P < 0.05); and lower than spring, summer and autumn in women (P < 0.05; **Figure 4.3C).** The prevalence of secondary hepatitis B vaccination response was lower in winter than summer in men and women (44% *vs.* 62%, χ^2 = 6.1, P <0.05, **Figure 4.3D**).

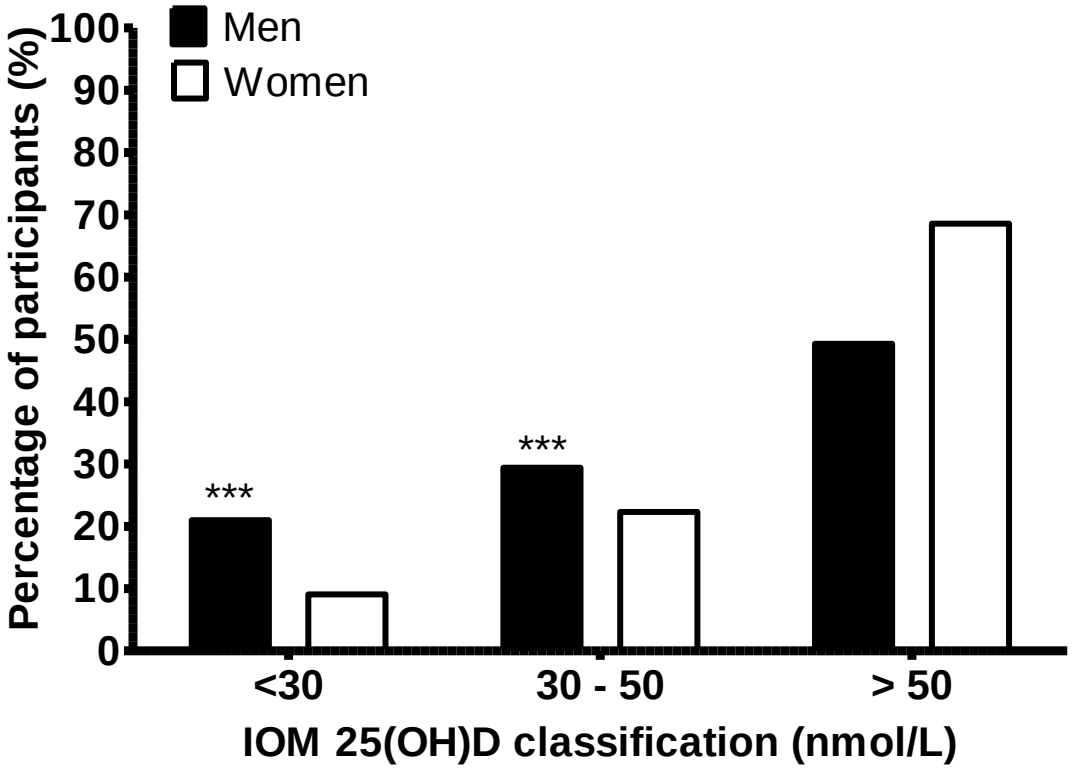

Figure 4.2. Percentage of participants categorized as vitamin D deficient (serum 25(OH)D <30 nmol/L [n(%)], men = 58 (21), women = 16 (9)), vitamin D insufficient (serum 25(OH)D 30 – 50 nmol/L [n(%)], men = 80 (29), women = 39 (22)), and vitamin D sufficient (serum 25(OH)D ≥ 50 nmol/L [n(%)], men = 134 (49), women = 120 (70)), in 447 healthy, young men (n = 272) and women (n = 175) residing in the UK. *** P < 0.001, greater than women.

Table 4.1. Participant demographics, anthropometrics, lifestyle characteristics and physician diagnosed 'all-cause illness.'

	Winter		*Spring*		*Summer*		*Autumn*	
	Men n = 54	Women n = 34	Men n = 32	Women n = 31	Men n = 93	Women n = 22	Men n = 93	Women n = 88
Demographics								
Age (years)	21.4 ± 3.0	21.8 ± 3.1	22.3 ± 3.1	21.9 ± 3.3	21.5 ± 2.5	23.5 ± 4.3	21.0 ± 2.8*	22.1 ± 3.2
Ethnicity, Caucasian [*n* (%)]	54 (100)	31 (91)	31 (97)	31 (100)	88 (95)	21 (96)	90 (97)	88 (100)
Anthropometrics								
Height (cm)	1.8 ± 0.1**	1.6 ± 0.1	1.8 ± 0.1**	1.6 ± 0.1	1.8 ± 0.1**	1.6 ± 0.1	1.8 ± 0.1**	1.6 ± 0.1
Body mass (kg)	77.2 ± 9.2**	64 ± 9.8	76.3 ± 10.2**	65.0 ± 8.1	76.3 ± 9.9**	64.4 ± 8.9	75.4 ± 10.4**	65.2 ± 7.6
BMI (kg/m^2)	24.4 ± 2.8*	23.2 ± 2.8	24.5 ± 2.9	24 ± 2.5	24.2 ± 2.6	23.4 ± 2.5	24.1 ± 2.9	23.7 ± 2.4
Lifestyle behaviors								
Alcohol user, [*n* (%)]	48 (89)	28 (82)	26 (81)	24 (77)	79 (85)	20 (91)	80 (86)	71 (81)
Smoker, [*n* (%)]	35 (65)	18 (53)	23 (72)	15 (49)	60 (64)	11 (50)	62 (67)	35 (40)
Sleep night before initial vaccination								
Duration (h)	6.3 ± 0.7	6.3 ± 0.6	6.6 ± 0.4**	6.2 ± 0.5	6.4 ± 0.7*	5.6 ± 1.5^{cd}	6.7 ± 0.9*	6.4 ± 0.8
Quality (very poor =1 to very good =4)	2.0 ± 0.6**	1.5 ± 0.8	1.8 ± 0.7	1.5 ± 0.8	1.9 ± 0.7*	1.3 ± 0.9	1.9 ± 0.7**	1.4 ± 0.8
Mood before initial vaccination								
Depression	0.3 ± 0.7*	0.8 ± 1.3	0.9 ± 2.8	0.3 ± 0.7	0.7 ± 1.3	0.4 ± 1.2	1.0 ± 1.7	0.6 ± 1.3
Fatigue	3.1 ± 2.1	4.1 ± 3.3	4.8 ± 4.0	3.9 ± 2.4	4.2 ± 2.9	4.1 ± 2.4	4.6 ± 3.0	4.2 ± 3.0
Tension	2.6 ± 2.2***	5.2 ± 3.2	4.2 ± 3.5	5.2 ± 4.0	3.9 ± 2.8*	5.8 ± 3.6	4.8 ± 3.6*	5.9 ± 3.3
All Cause Illness [*n* (%)]	6 (11)	2 (6)^{c}	7 (22)	3 (10)	6 (7)^{ac}	4 (18)	22 (24)	21 (24)

Study-1 seasonal and between sex comparisons of baseline participant demographics, anthropometrics, and lifestyle behaviours (n = 447; 272 men, 175 women). All cause illness includes; physician diagnosed upper respiratory tract infections, lower respiratory tract infections and gastrointestinal infections. Values presented as mean ± SD unless otherwise stated. [a] $P < 0.05$ lower than spring, [b] lower than summer, [C] $P < 0.05$ lower than autumn. [D] $P < 0.05$ lower than winter. * $P < 0.05$, ** $P < 0.01$, indicates significant difference between men and women.

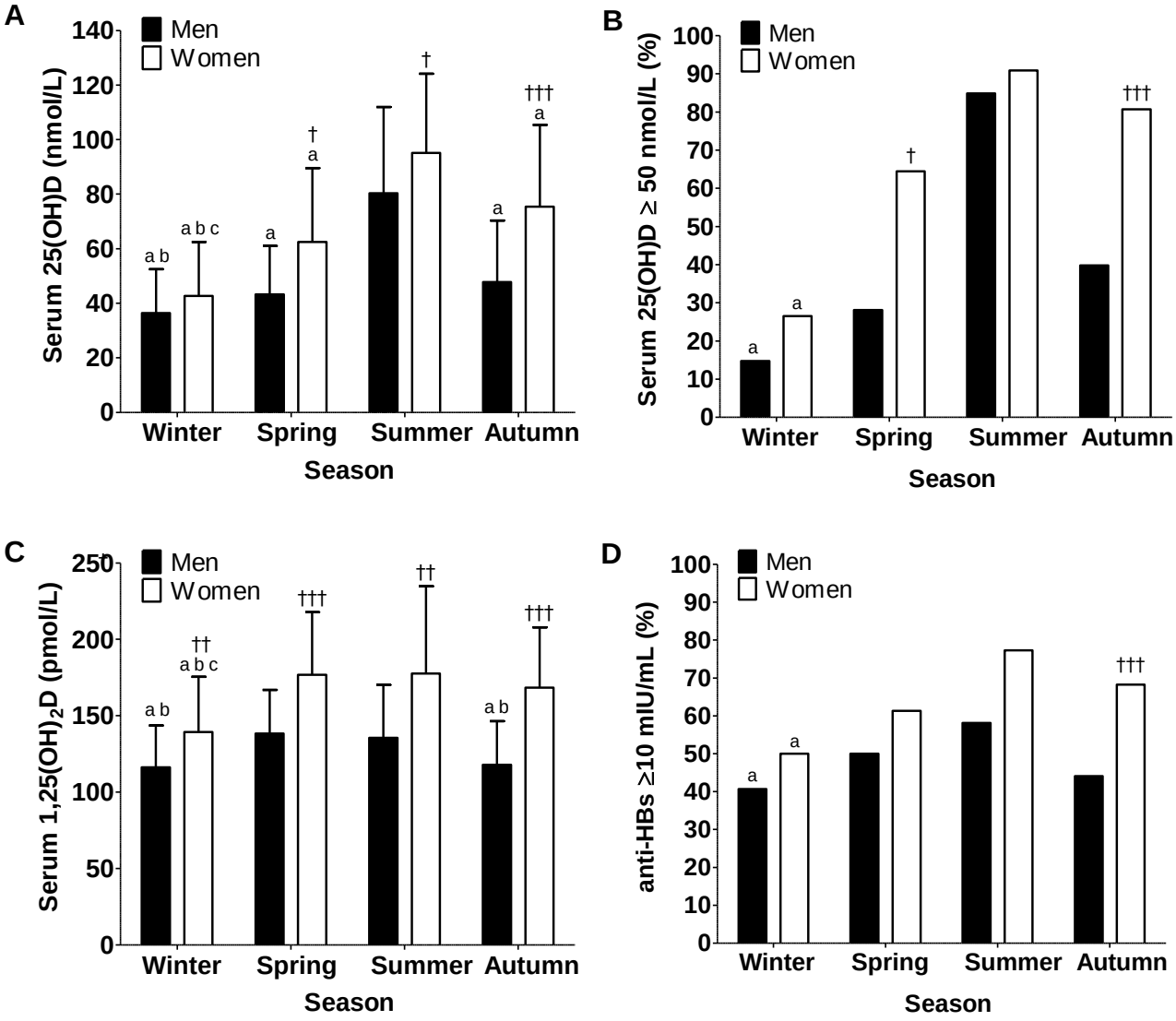

Figure 4.3. Seasonal variation in serum 25(OH)D (panel A), percentage of participants categorized as vitamin D sufficient (25(OH)D ≥ 50 nmol/L; panel B), serum 1, $25(OH)_2D$ (panel C), and percentage of secondary hepatitis B vaccination responders (anti-HBs ≥ 10 mIU/mL; panel D) in 447 healthy, young men (*n* = 272) and women (*n* = 175) residing in the UK. Panel A and C data are mean ± SD. Panel B and D are percentages represented by vertical bars. a, lower than summer ($P < 0.05$). b, lower than autumn ($P < 0.05$). c, lower than spring ($P < 0.05$). † $P < 0.05$, †† $P < 0.01$ and ††† $P < 0.001$, greater than men.

Higher vitamin D status and hepatitis B vaccine response. When directly examining the relationship between vitamin D and secondary hepatitis B vaccine response, **Figure 4.4A**

shows a trend for a lower percentage of men and women with insufficient vitamin D status at the time of initial vaccination being vaccine responders, compared to those with sufficient vitamin D status (50% *vs.* 59%; $P = 0.09$). However, when separated, fewer men with insufficient vitamin D status (43%) at the time of initial vaccination were vaccine compared to men with sufficient vitamin D status (55%) at the time of initial vaccination ($\chi^2 = 3.7$; $P = 0.05$, **Figure 4.4B**). Also, in a logistic regression, vitamin D insufficient men were less likely to be hepatitis B vaccine responders than vitamin D sufficient men (OR 1.6; 95% CI 1–2.6; $P < 0.05$). Secondary anti-HBs response was not different among women displaying vitamin D sufficiency or insufficiency at the time of initial vaccination (**Figure 4.4B**). In addition, fewer men and women categorized in the lowest $1,25(OH)_2D$ tercile were vaccine responders compared to those in the highest $1,25(OH)_2D$ category (Q1 *vs.* Q3; 50% *vs.* 62%; $P < 0.05$, **Figure 4.4C**). When separated, secondary anti-HBs response was not different across the 1, $25(OH)_2D$ categories in men or women ($P > 0.05$, **Figure 4.4D**).

4.5 DISCUSSION

In a prospective cohort study of 447 young healthy persons, completed across all seasons, we showed that fewer men and women who received their initial hepatitis B vaccination during the winter mounted a response to the hepatitis B vaccination (anti-HBs titres ≥10 mIU/mL) in comparison to men and women vaccinated during summer months. This seasonal variation in secondary anti-HBs response mirrored the typically observed seasonal variation in serum 25(OH)D concentrations as well as the higher prevalence of vitamin D insufficiency during the winter. We also demonstrate a seasonal variation in the biologically active form of vitamin D, serum 1, $25(OH)_2D$. In our primary analyses we also show direct evidence that vitamin D might be an important influencing factor for the development of hepatitis B vaccination response. Indeed, fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations (**Figure 4.4**). These associations were particularly stronger in men compared to women, likely due to a greater prevalence of vitamin D insufficiency in men compared to women (serum 25(OH)D ≥ 50 nmol/L; men *vs.* women, 49% *vs.* 70%, **Figure 4.2**). These findings coupled with fewer individuals achieving sufficient vitamin D status (25(OH)D and $1,25(OH)_2D$) and responding to hepatitis B vaccination during the winter (15% of men and 26% of women; F**igure 4.3B**) may highlight the need for vitamin D supplementation during the winter.

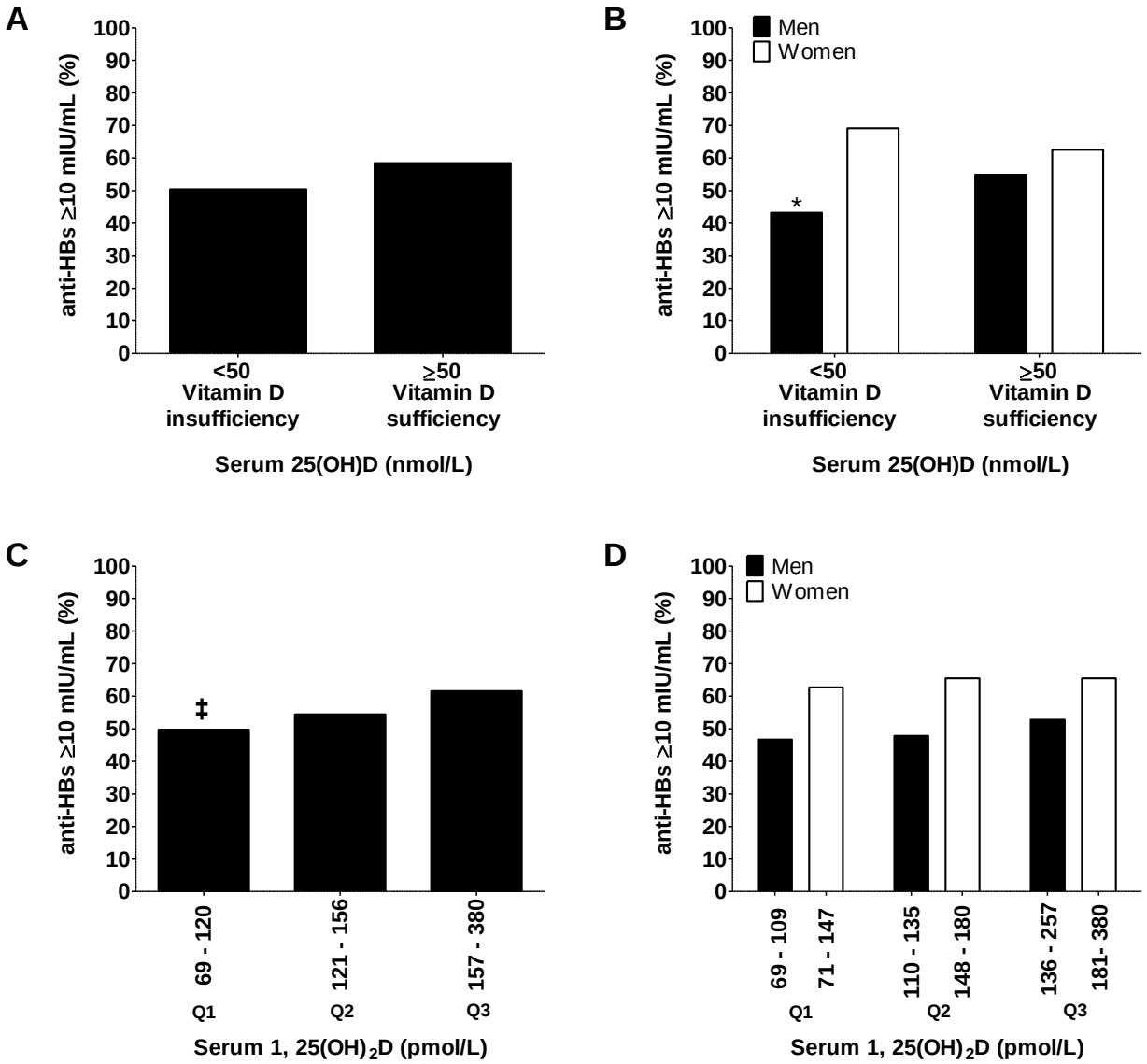

Figure 4.4. Percentage of secondary hepatitis B vaccine responders (antibody ≥ 10 mIU/mL) across vitamin D insufficient (< 50 nmol/L) and sufficient (≥ 50 nmol/L) men and women combined (Panel A) and separated (Panel B), and across serum 1,25OH_2D terciles, combined (Panel C) and separated (Panel D). Panel A, and B present data for 272 men (< 50 nmol/L = 139, ≥50 nmol/L = 133) and 175 women (< 50 nmol/L = 55, ≥50 nmol/L = 120). Panel C and D present data for 269 men and 175 women (Panel C; Q1 = 148 [men = 124, women = 24], Q2 = 148 [men = 99, women = 49], Q3 = 148 [men = 46, women = 102]) and separated (Panel D: men: Q1 = 90, Q2 = 90, Q3 = 89; women Q1 = 59, Q2 = 58, Q3 = 58). * $P < 0.05$, lower percentage of secondary hepatitis B vaccination responders (anti-HBs ≥ 10 mIU/mL) in vitamin D insufficient men than vitamin D sufficient men. ‡ < 0.05, lower percentage of secondary hepatitis B vaccination responders (anti-HBs ≥ 10 mIU/mL) in Q1 compared to Q3.

Several factors are known to contribute to an inadequate hepatitis B vaccine response in otherwise healthy young adults, including; male sex, BMI ≥ 25, smoking, poor sleep and psychological stress (Averhoff et al., 1998; Glaser et al., 1992; Prather et al., 2012; Roome et al., 1993; Westmoreland et al., 1990; Wood, MacDonald, et al., 1993; Yang et al., 2016). However, a particular strength of the current study was the relative homogeneity in these factors both between sexes and across seasons (Table 4.1). We add to the current literature by demonstrating how vitamin D may play an important role in the development of antibody response following hepatitis B vaccination in young and otherwise healthy individuals. Indeed, fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations, compared to those with vitamin D sufficiency and higher $1,25(OH)_2D$ concentrations. Further, both women and men who were vaccinated during the winter, when serum 25(OH)D and $1,25(OH)_2D$ were lowest, had a response rate lower than summer and compared to what is normally expected following 2 out of the 3 hepatitis B vaccine doses (>50%) (Joines et al., 2001). The potential role of vitamin D may be mediated via its direct interaction with antigen presenting cells and more particularly with dendritic cells. These roles may include dendritic cell activation, via TLRs by vaccine antigen, and intracellular upregulation of CYP27B1 with the induction of cytokine production and T cell proliferation (Lang & Aspinall, 2015). Furthermore, animal studies have shown that $1,25(OH)_2D$ induced dendritic cell migration to stimulate T and B-cell antibody response to diphtheria vaccination (Enioutina et al., 2008, 2009).

In agreement with previous literature, we observed that women responded better than men to the hepatitis B vaccination. The specific details about the mechanisms mediating how men and women differ in response to vaccination are lacking (Klein, Marriott, & Fish, 2015); however, it has been previously been suggested that superior response to vaccination among women may be owing to the opposite effects of sex hormones. Specifically, oestrogen has been implicated to promote antibody production, whilst androgens exhibit inhibitory effects on antibody production by B cells (Furman et al., 2014; Lu et al., 2002). However, greater vaccination responses observed in pre-pubertal girls compared to boys, and post-menopausal women compared to elderly men suggest more inherent genetic and microbiota differences between women and men may better explain the sex based differences in vaccination response (Klein et al., 2015). In line with our current findings, greater anti-HBs response among women compared to men might also be explained by women, on average and irrespective of season, having higher serum vitamin D concentrations (25(OH)D and $1,25(OH)_2D$).

It should be acknowledged that differing seasons of the year and UVB exposure are factors that determine vitamin D status, may also elicit vitamin D independent immunomodulation. For instance, seasonal variation in host immunity. Infectious disease and host immunity are mutually linked so while depressed immunity increases the risk of clinical infection in those exposed to pathogens, infection itself can debilitate the host, resulting in reduced immune defences. Therefore, some infectious diseases driven by external factors, including; climate and variation in sunshine levels could debilitate the host and in-turn predispose to secondary infection; resulting in seasonality of the secondary infection. A particularly well documented example is that of diarrheal illness in children; predisposing them to subsequent pneumonia (Ashraf, Huque, Kenah, Agboatwalla, & Luby, 2013; Schmidt, Cairncross, Barreto, Clasen, & Genser, 2009). However, in the case of the current study we show no evidence of this as 'all cause illness,' consisting of physician diagnosed URTI, LRTI and gastrointestinal infections, were not different between winter and summer (Table, 4.1).

We recognise that final antibody status following the full three-dose hepatitis B vaccine course was not captured in the current study. Methodologically, we chose to examine the influence of vitamin D on hepatitis B vaccine response following the second dose of the typical three dose series because there is widespread inter-individual variability in the magnitude of antibody response at this time (Szmuness et al., 1980). In addition, the range of anti-HBs response following the second vaccine dose provides an experimental model to explore and identify factors which may influence the development of antibody response. In comparison, ~90% of individuals are considered to be sero-protected (anti-HBs ≥10 mIU/mL) following the third and final hepatitis B vaccine dose (Prather et al., 2012). Regardless, future studies may seek to confirm the importance of vitamin D status at the time of initial vaccination on sero-protective antibody status following the full hepatitis B vaccine course. Given that the present study has demonstrated that fewer individuals who had low vitamin D status (25(OH)D and 1,25$(OH)_2$D) mounted a response to the hepatitis B vaccination in comparison to individuals vaccinated with higher vitamin D status (**Figure 4.4**). It could be postulated that these findings may also have implications that extend to other populations at greater risk of low vitamin D status; including the diseased and elderly. Indeed, it has been shown that vitamin D deficiency is highly prevalent in elderly CKD patients (Zitt et al., 2012). The same study also demonstrated that patients with vitamin D deficiency were more frequently non responders to the hepatitis B vaccination in comparison to patients who were not vitamin D deficient (Zitt et al., 2012).

In conclusion, fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations. Of particular clinical interest is that vaccine responses in vitamin D insufficient men were lower than typically expected after two hepatitis B vaccine doses. This direct association between vitamin D and successful hepatitis B vaccination might explain the seasonal variation in hepatitis B vaccine response also observed in the current study. Specifically, fewer men and women who received their initial hepatitis B vaccination during the winter mounted a response to the hepatitis B vaccination in comparison to men and women vaccinated during summer months. Based on the current evidence we suggest a need for vitamin D supplementation during winter months, given the compromised anti-HBs response following vaccination and low vitamin D concentrations, particularly in men, during the winter.

CHAPTER FIVE

The Influence of oral vitamin D_3 supplementation and Simulated Solar Radiation (SSR) on serum vitamin D

5.1 SUMMARY

Eighty percent of young healthy adults residing at northern latitudes fail to meet the European Food Safety Authority (EFSA) and Institute of Medicine (IOM) recommendations to maintain serum 25(OH)D concentrations ≥ 50 nmol/L during the winter. However, no studies have sought to correct wintertime nadirs in vitamin D concentrations and promote vitamin D sufficiency; using either simulated sunlight (in accordance with recommendations on safe sunlight exposure) or government guided oral vitamin D_3 doses. The current study aimed to compare the influence of raising and maintaining vitamin D (serum 25(OH)D) using methods that replicate ecologically valid simulated doses of sunlight exposure (SSR) and safe and government recommended oral doses of vitamin D_3. Twenty-eight healthy recreationally active males and females (mean ± SD: age 20 ± 2 years, height 177 ± 8 cm, body mass 73.4 ± 10.7 kg) were initially randomly allocated to one of four groups; 4-week 'restoration' phase of either thrice weekly solar stimulated radiation (SSR), 1,000 IU/day oral vitamin D_3 capsules (ORAL), or matched placebos; SSR placebo (SSR-P) and Oral placebo (ORAL-P). Then during a further 8-week maintenance period, 50% of participants in each of the experimental groups (SSR and ORAL) went onto receive a maintenance dose of once weekly SSR exposures or 400 IU per day vitamin D_3. The remaining 50% of each of these groups were transferred to the placebo treatments for the remaining 8 weeks. Those originally allocated to the SSR-P or ORAL-P groups remained on placebo regimens. Blood samples were collected for the assessment of serum 25(OH)D before starting the intervention, at week 5 following the restoration phase, and week 9 and 12 of the maintenance phase. Mixed-model ANOVA demonstrated that four weeks of thrice weekly SSR exposures and 1,000 IU/day increased 25(OH)D similarly and higher than the respective placebo forms (mean ± SD, SSR *vs*. SSR-P = 59 ± 10 *vs*. 35 ± 17 nmol/L; $P < 0.05$, ORAL *vs*. ORAL-P = 74 ± 11 *vs*. 29 ± 19 nmol/L; $P < 0.01$). Maintenance doses of either the once weekly SSR exposure or 400 IU daily vitamin D_3 resulted in a further increase in serum 25(OH)D. In conclusion, the results from the present study suggest safe and practical government recommended summer sunlight (SSR) and daily oral vitamin D_3 supplementation guidelines administered over 12 weeks similarly restored and maintained 25(OH)D sufficiency and increase concentrations to 'typical' summer concentrations.

5.2 INTRODUCTION

During winter, at latitudes above 43°N, cutaneous synthesis of vitamin D via sunlight contributes little to serum vitamin D (serum 25(OH)D) production; leading to a distinct seasonal pattern of serum 25(OH)D (Webb et al., 1988), which is also demonstrated in **Chapter 4** of this thesis. This seasonal pattern is characterised by > 80% of healthy young adults achieving vitamin D sufficiency (serum 25(OH)D ≥ 50 nmol/L) during the summer, compared to < 30% during the winter. Avoiding low serum 25(OH)D is essential for bone and general health; with recent European Food Safety Authority (EFSA) and Institute of Medicine (IOM) recommendations to maintain serum 25(OH)D concentrations ≥ 50 nmol/L (European Food Safety Authority, 2016; Institute of Medicine, 2011). In recent years, vitamin D has been shown to be important for various aspects of immune function and protection against respiratory infection (He, Aw Yong, et al., 2016). To date no thresholds or classifications regarding vitamin D status in relation to immune health exist. However, findings from **Chapter 4** suggest that fewer vitamin D insufficient individuals (serum 25(OH)D < 50 nmol/L), especially men, mounted a secondary anti-HBs response (anti-HBs ≥ 10 mIU/mL), in comparison to those with sufficient vitamin D status at the time of initial vaccination. Research using randomised-controlled trials of vitamin D supplementation are required to substantiate this purported 50 nmol/L cut off for serum 25(OH)D for developing optimal *in vivo* immune responses to novel markers such as the hepatitis B vaccine. Before any causal relationships can be established, appropriate prospective randomised trials that seek to restore and maintain vitamin D sufficiency during winter are required.

Two main methods which can be adopted to avoid seasonal nadirs in vitamin D concentrations, are firstly; taking a daily oral vitamin D_3 supplement during the winter and secondly, where possible, practicing safe summer sunlight exposure is important to optimise vitamin D status (He, Aw Yong, et al., 2016). Although, past investigations have compared the efficacy of raising 25(OH)D via artificial narrow-band, ultra-violet B (NB-UVB) exposure versus oral vitamin D_3 supplementation (Ala-Houhala et al., 2012; Bogh, Gullstrand, Svensson, Ljunggren, & Dorkhan, 2012). No studies have compared the increase in serum 25(OH)D as a result of ecologically valid simulated doses of sunlight exposure to safe and government recommended oral doses of vitamin D_3. These findings may have important methodological implications for future studies that seek to investigate differential effects of these safe and

government guided supplementation methods on any clinical endpoints i.e. the secondary antibody response to vaccination.

Exposure to UVB radiation in sunlight is essential for cutaneous synthesis of vitamin D, and is the body's main source of vitamin D (Farrar et al., 2013). Advisory boards, including the United Kingdom's Health Protection Agency, in keeping with other countries positioned at similar latitudes, advise that casual exposures to summer sunlight, contain the required UVB to attain recommended vitamin D status (National Radiological protection board, 2002). However, there has been uncertainty regarding the specification and impact of following this guidance. Contrary to this, using a carefully characterised radiation cabinet to mimic typical casual UK summer sun exposure during winter, Rhodes and colleagues were able to restore 25(OH)D sufficiency in ≥ 90% of individuals, without causing skin erythema, among a white population based in the United Kingdom (Rhodes et al., 2010). However, it is unknown whether this regimen adequately maintains vitamin D sufficiency during the winter months when there is limited or no natural UV exposure. Further, the dose per week of solar simulated radiation (SSR) exposures as in Rhodes et al. (2010) to maintain 25(OH)D sufficiency following a period of restoration remains to be established.

Alternatively, oral supplementation with vitamin D_3 is an effective method to increase serum 25(OH)D (Houghton & Vieth, 2006). Serum 25(OH)D responds to supplementation in a dose dependant manner (Heaney, Davies, Chen, Holick, & Barger-Lux, 2003). As such, experimenters favour incorporating oral vitamin D_3 supplement into the design of a study in order to control the magnitude of 25(OH)D increase. European food safety authority and the U.S. Institute of Medicine (IOM) recommend a safe daily upper limit of 4,000 IU/day (Institute of Medicine, 2011). However, much lower oral doses of vitamin D_3 including 1,000 IU/day have been shown to be effective in increasing serum 25(OH)D to concentrations ≥ 50 nmol/L (Macdonald et al., 2013; Vieth, Chan, & MacFarlane, 2001). Crucially, this supplemental dose appears to causes a similar change in serum 25(OH)D as the SSR protocol (~30 nmol/L), developed by Rhodes and colleagues. As with the SSR protocol developed by Rhodes & colleagues, how long individuals are able to maintain restored vitamin D status after frequent oral vitamin D_3 ingestion remains to be established. Limited evidence from older adults (65 years and over) indicates that an intake of 400 IU/day maintains 25(OH)D concentrations of 25-100 nmol/L in cases of minimal endogenous synthesis (MacLennan & Hamilton, 1977; Toss, Larsson, & Lindgren, 1983). Despite IOM recommendations supporting 400 IU/day as

the estimated average requirement for adults aged between 18-50 years of age, there are no randomised trials in this age group to support this.

The primary aim of the study was to compare the magnitude of change in serum 25(OH)D incurred by 3 times weekly SSR with a dose of oral vitamin D_3 (1,000 IU/day) over a period of 4 weeks. Secondly, to understand how less frequent SSR exposures (once-a-week) and a reduced oral dose (400 IU/day; March–May) for a further 8 weeks influence 25(OH)D concentrations in a young healthy population. The findings of this study would help to inform/optimise vitamin D supplementation methods to achieve EFSA and IOM recommendations to maintain serum 25(OH)D concentrations ≥ 50 nmol/L, which is of particular importance for optimal bone and general health, and potentially for immune function.

5.3 METHODS

Participants. Thirty-three healthy recreationally active males and females (mean ± SD: age 20 ± 2 years, height 177 ± 8cm, body mass 73.4 ± 10.7 kg) volunteered to participate in this study conducted at the School of Sport, Health and Exercise Sciences (SSHES), Bangor University (Latitude 53°N). The recruitment for this study took place in February 2015 and testing commenced in the same month. The study subsequently concluded in May 2015.

Baseline measurements. Volunteers were screened for various inclusion/exclusion criteria as outlined in **Chapter 3, section 3.2**. Height and body mass measurements were collected from eligible volunteers as described in **Chapter 3, section 3.3**. Participants completed a food frequency questionnaire (FFQ) to assess typical dietary vitamin D before enrolling in the study.

Supplementation.

Restoration phase (Weeks 1-5). Participants were randomly allocated to one of four groups; oral vitamin D_3 supplementation (ORAL; 1000 IU/day), oral placebo (ORAL-P), solar simulated radiation (SSR), or SSR placebo (SSR-P). Block randomisation was used to account for differences in sex and BMI. Participants were blind to treatments; investigators were blind to ORAL treatments but not for SSR. Participants in the ORAL and ORAL-P groups were required to report to the laboratory on a weekly basis to collect their ORAL or placebo supplement for a period of four weeks. Compliance to the ORAL supplements at the end of the

restoration phase was estimated by counting the unused capsules at each subsequent visit. Those assigned to the SSR intervention were exposed three-times-a-week during the restoration phase to an investigator controlled constant UVR dose using a whole body irradiation cabinet (protocol described in **Chapter 3, section 3.7.**

Maintenance phase (Weeks 5-12). Immediately following the restoration phase, 50% of participants who were initially randomised to ORAL received a 400 IU/day maintenance dose, and the other 50% received placebo capsules for 8 weeks. Those originally allocated to the ORAL-P groups remained taking the placebo capsules for a further 8 weeks. As with the restoration phase, compliance to the ORAL supplements at the end of the 8-week maintenance phase was estimated by counting the unused capsules at each subsequent visit. Fifty percent of participants who were initially randomised to SSR treatment went onto a reduced dose of SSR once-a-week whilst the other 50% moved into the SSR-P group and were exposed once a week for a further 8 weeks. Those originally allocated to the SSR-P groups remained on the placebo cabinets and were exposed once a week over the 8-week maintenance period. All participants receiving oral supplementation, ORAL or ORAL-P, were required to report to the laboratory on a weekly basis to collect their oral or placebo supplement for a period of eight weeks. On completion of the study, participants were asked to guess which intervention they though they has been receiving during the restoration and the maintenance phases of the study. A schematic for trial events is summarised in **Figure 5.1**.

Blood sampling. In order to monitor the change in vitamin D status, blood samples for the assessment of serum concentrations of serum 25(OH)D, were drawn from the participants before supplementation (week 1), and then at weeks 5, 9 and 12 (collection method and sample preparation described in **Chapter 3, section 3.6**. Blood samples were collected in the morning at a similar time of day, and were collected following five minutes of seated rest by trained phlebotomists.

Sample size and statistical analysis. Sample size was estimated using the change in 25(OH)D incurred by supplementation with 1,000 IU/day of vitamin D_3 *vs.* placebo from a previous vitamin D supplementation study (Macdonald et al., 2013). With the alpha level set at 0.05, and power 0.95 (G*Power software, version 3.1.2), a sample size of 6 was calculated to be necessary to detect a significant difference between two dependent means. Therefore, six

participants per treatment group was required to detect a meaningful difference in 25(OH)D. Comparisons of baseline demographic characteristics and serum 25(OH)D concentration for the four groups were made using a one way analysis of variance (ANOVA) and Kruskal Wallis for normally and non–normally distributed variables respectively. A 4 x 2 mixed model ANOVA was used to compare all groups across the restoration phase (week 1 to week 5). Then an additional 6 x 3 mixed model ANOVA was used to compare groups across the maintenance period (weeks 4, 9 and 12). Where significant main or interaction effects were observed, post-hoc pairwise comparisons were conducted using Tukey's post hoc test.

5.4 RESULTS

Participant flow and characteristics. Of the 33 volunteers who were recruited and completed the restoration phase of the study, 28 participants completed both the restoration and maintenance phases of the intervention. Two participants withdrew from the study, 1 from the SSR-P group and 1 from the ORAL-P group during the maintenance phase of the study for reasons unrelated to the intervention. A further three were excluded due to missing data. The remaining 28 had all four-blood samples taken and were included in the final analysis. The baseline characteristics of the two groups are shown in **table 5.1**. Mean age, body mass index, Fitzpatrick skin type, serum 25(OH)D concentrations at baseline and estimated vitamin D intake were all similar between the treatment groups.

Participant blinding questionnaires. Overall compliance was ≥ 80% for all groups across both phases of the study. The success of the blinding was achieved with 57% and 50% of participants guessing correctly, as to which group they were in during the restoration and maintenance phases respectively.

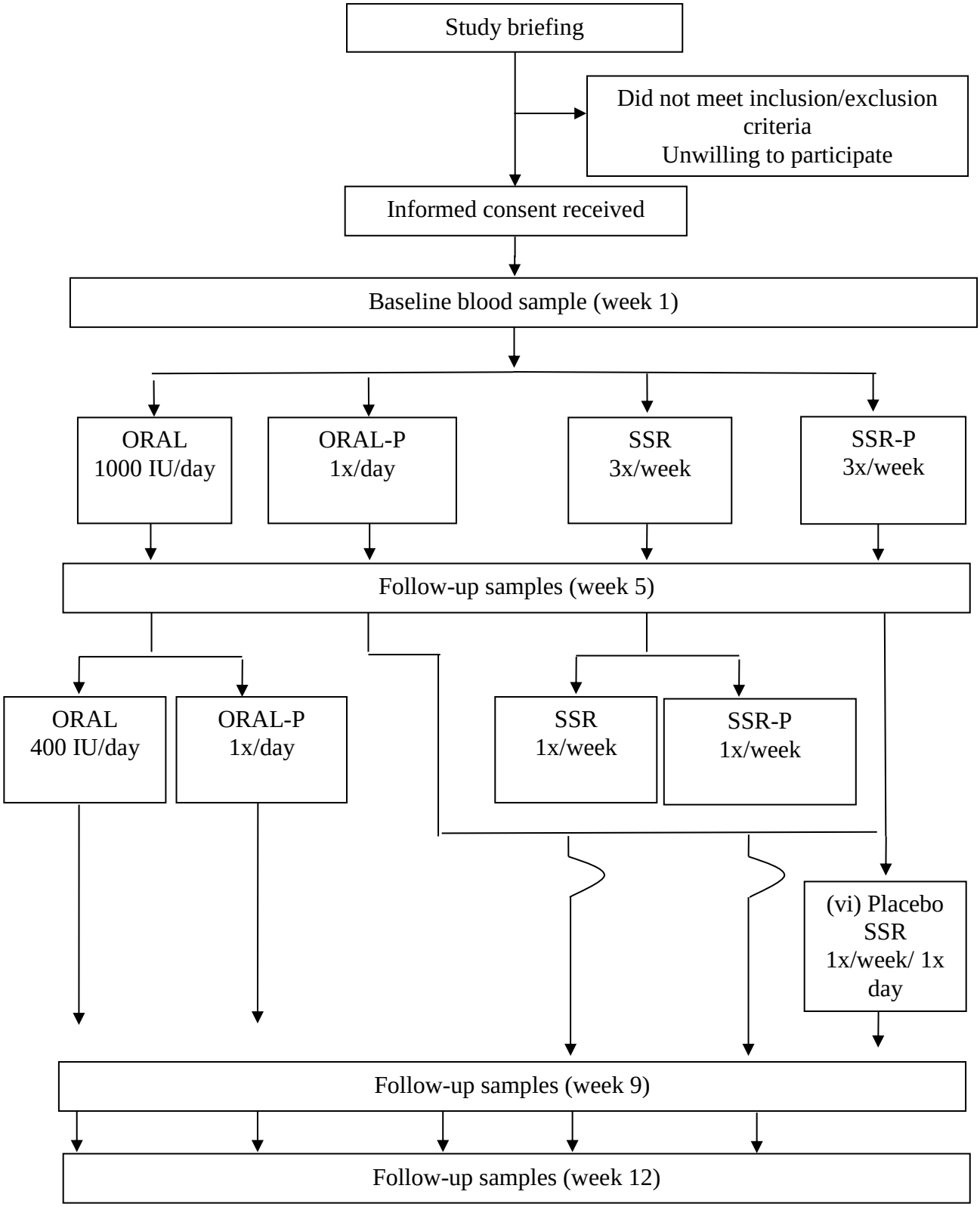

Figure 5.1. Schematic of trial events. SSR; solar simulated radiation, ORAL; oral vitamin D_3, SSR-P; solar simulated radiation placebo, ORAL-P; oral vitamin D_3 placebo.

Table 5.1. Participant demographics and baseline serum 25(OH)D concentration across intervention groups; solar simulated radiation (SSR), SSR placebo (SSR-P), oral vitamin D_3 (ORAL), oral placebo (ORAL-P).

	ORAL (n=6)	ORAL-P (n=6)	SSR (n=10)	SSR-P (n=6)	*P* value
Demographics					
Male/female	5/1	5/1	8/2	4/2	-
Age (years)	19.8 ± 1.0	19.7 ± 1.0	21.2 ± 3.0	19.2 ± 0.7	0.2
Skin type (I, II, II, IV)	0, 2, 4, 0	0, 2, 3, 1	1, 3, 6, 0	0, 2, 3, 1	
Anthropometrics					
Height (cm)	181 ± 5	179 ± 3	175 ± 10	174 ± 12	0.5
Body mass (kg)	73 ± 11	71 ± 5	72 ± 12	75 ± 13	0.9
Body mass index (kg/m^2)	22.3 ± 3.4	22.4 ± 1.6	23.5 ± 2.3	24.8 ± 4.0	0.4
Serum assessments - Week 1					
Serum 25(OH)D nmol/L (range)	35 (26 – 51)	27 (13-59)	29 (14-43)	39 (23-58)	0.4
Dietary vitamin D intake μg/day (range)	2.5 (1.3-3.5)	2.8 (0.7-5.1)	1.9 (0.7-3.0)	3.2 (0.9-10.4)	0.6

Restoration phase; serum 25(OH)D response. Four weeks of SSR and ORAL supplementation were successful strategies to restore 25(OH)D concentrations so that at week 5 serum 25(OH)D concentrations in the SSR and ORAL groups were higher than their respective placebos ($P < 0.001$) (**Figure 5.2A and B**). The magnitude of serum 25(OH)D concentration change was matched between the SSR and ORAL groups (mean ± SD; ORAL = 39 ± 11 nmol/L, SSR = 30 ± 10 nmol/L; P=0.2). Serum 25(OH)D remained unaltered in the placebo groups during the restoration phase. At week 1, 86% of participants presented with vitamin D insufficiency (serum 25(OH)D < 50 nmol/L), in accordance with the Institute of medicine (IoM) guidelines (Institute of Medicine, 2011). Following the restoration phase, 100% and 80% of participants achieved sufficient 25(OH)D (≥ 50 nmol/L) in the ORAL and SSR groups, respectively (**Figure 5.3A and B**). In comparison, only 33% and 17% of

participants displayed sufficient vitamin D status in the SSR-P and ORAL-P groups, respectively.

Maintenance phase: serum 25(OH)D response. Receiving maintenance doses of either once-a-week SSR exposures (SSR) or 400 IU/daily vitamin D_3 (ORAL) for a further 8 weeks during the maintenance phase resulted in a further increase of serum 25(OH)D ($P < 0.05$) (**Figure 5.2C and D**). By week 12, 100% of participants receiving vitamin D supplementation (SSR or ORAL) were vitamin D sufficient (**Figure 5.3C and D**). Serum 25(OH)D concentrations of participants receiving ORAL supplementations were higher compared to those in the ORAL-P group at week 9 ($P < 0.05$). Comparatively, 25(OH)D concentrations were similar between those in the SSR group compared to SSR-P participants. Participants who remained in the placebo groups throughout the study had an increase in serum 25(OH)D during the maintenance phase ($P < 0.01$). By week 12, all groups had similar serum 25(OH)D concentrations. However, only 50% and 33% of individuals in the SSR-P and ORAL-P groups, respectively, achieved vitamin D sufficiency by week 12. Participants who were transferred to placebo groups, had no change in serum 25(OH)D between week 5 and 12 (ORAL to ORAL-P; P=0.4, SSR to SSR-P; P=0.8). However, it should be noted that not all of these participants were able to maintain the sufficient 25(OH)D status achieved during the restoration phase, with a drop in the percentage of those displaying sufficiency dropping from 100% to 67% and 100% to 80% in the ORAL and SSR groups, respectively (**Figure 5.3C and D**).

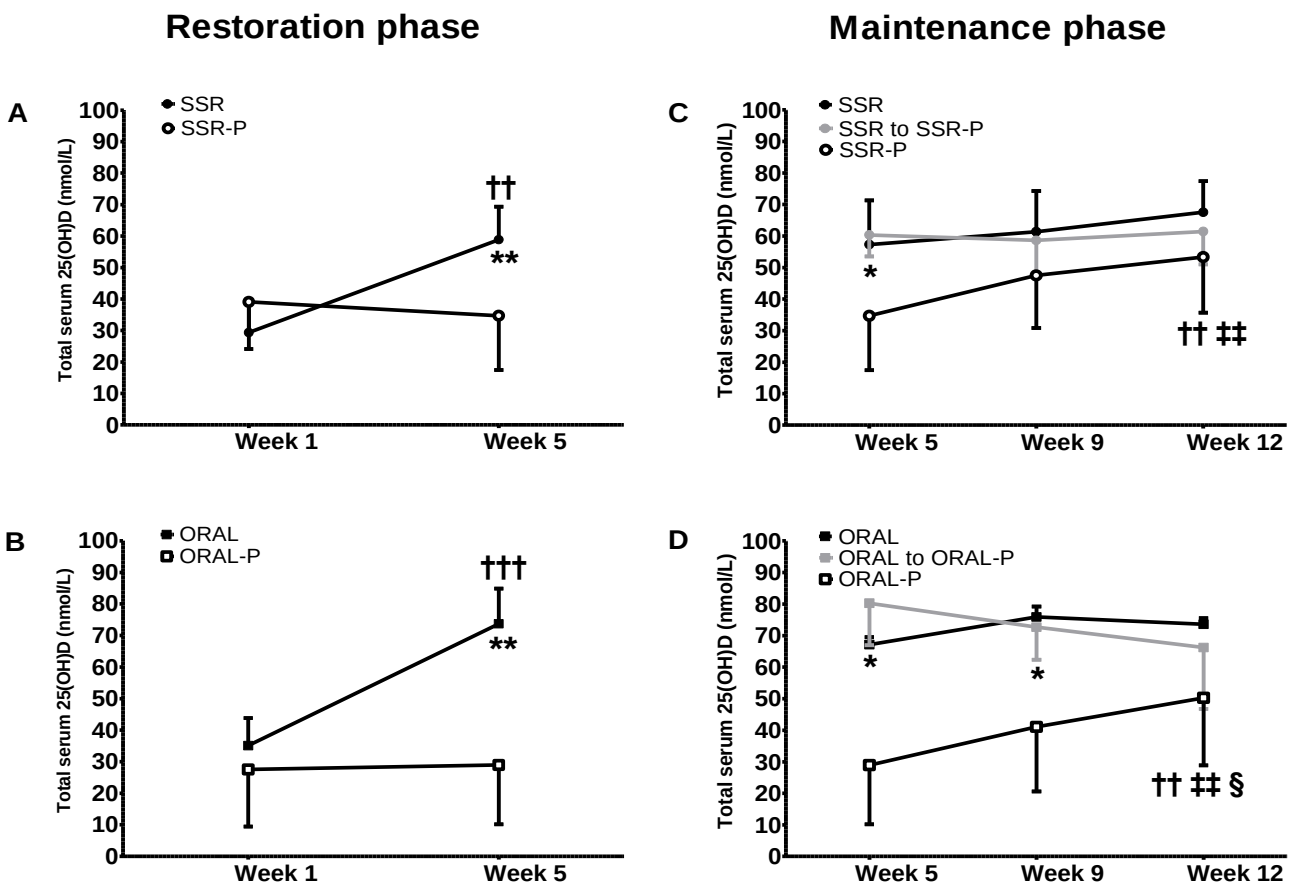

Figure 5.2. Serum 25(OH)D before and after 12 weeks of vitamin D supplementation by solar simulated radiation (SSR) and oral vitamin D_3 (ORAL). Panels **A** & **B** are solar simulated radiation (SSR) *vs*. SSR placebo (SSR-P) and oral vitamin D_3 (ORAL) *vs.* oral placebo (ORAL-P), respectively; during the restoration phase. Panels **C** & **D** are solar simulated radiation (SSR) *vs*. SSR placebo (SSR-P) and oral vitamin D_3 (ORAL) *vs.* oral placebo (ORAL-P), respectively during the maintenance phase. †† $P < 0.01$, greater than week 1. ‡‡ $P < 0.01$, greater than week 5. § $P < 0.05$, greater than week 9. * $P < 0.05$ and ** $P < 0.01$, greater than placebo. Data are mean ± SD.

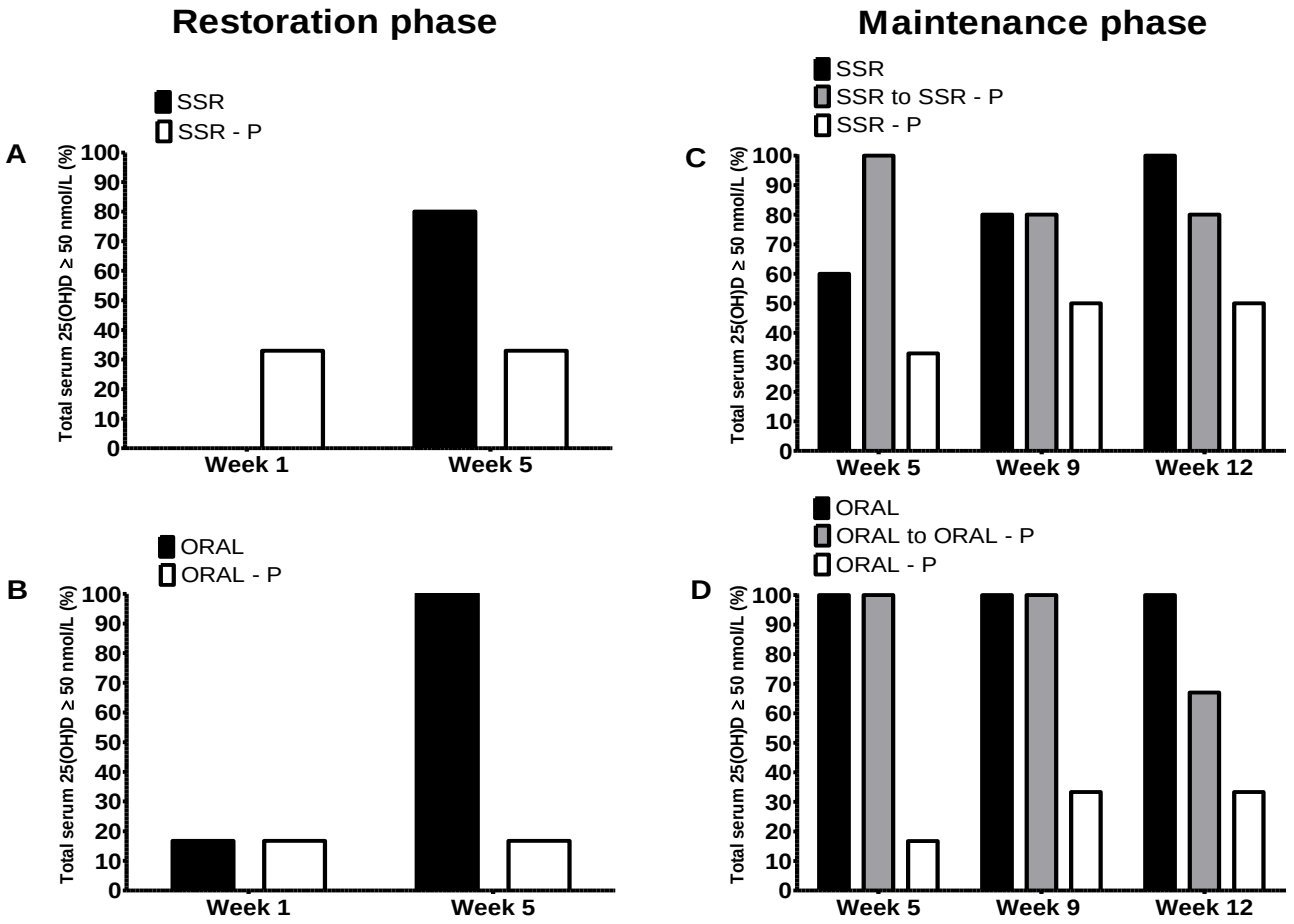

Figure 5.3. Vitamin D sufficiency (25(OH)D ≥ 50 nmol/L) before and after 12-weeks of vitamin D supplementation by solar simulated radiation (SSR) and oral vitamin D_3 (ORAL). Panels **A** & **B** are simulated solar radiation (SSR) *vs.* SSR placebo (SSR-P), and oral vitamin D_3 (ORAL) *vs.* oral placebo (ORAL-P), respectively during the restoration phase. Panels **C** & **D** are simulated solar radiation (SSR) *vs.* SSR placebo (SSR-P), and oral vitamin D_3 (ORAL) *vs.* oral placebo (ORAL-P), respectively during the maintenance phase. Vertical bars are percentages.

5.5 DISCUSSION

The aims of the study were firstly; to compare the magnitude of change in serum 25(OH)D incurred by three times weekly SSR with a dose of oral vitamin D_3 (1,000 IU/day) over a period of 4 weeks. Secondly, the study aimed to compare how less frequent SSR exposures (once-a-week) and a reduced oral dose (400 IU/day (March-May)) for a further 8 weeks influence 25(OH)D concentrations in a young healthy population. In this study we demonstrated that safe and practical government recommended summer sunlight (SSR) and daily oral vitamin D_3 supplementation regimens administered over 4 weeks could restore sufficiency in > 80% of participants and increase 25(OH)D to concentrations comparable to those observed during the summer. Importantly, SSR and ORAL treatments incurred similar changes in 25(OH)D. A further 8 weeks of once weekly SSR exposure and 400 IU/day maintained elevated serum 25(OH)D; and sufficient vitamin D status in 100% of participants.

In agreement with previous literature (Close, Leckey, et al., 2013; Close, Russell, et al., 2013; Morton et al., 2012), a large percentage of young healthy adults (> 80%) presented with vitamin D insufficiency at the start of the study; which commenced during a UK winter period. Our supplementation methods of thrice weekly exposure and 1,000 IU/day of vitamin D_3 for four weeks achieved EFSA and IOM defined vitamin D sufficiency in almost all participants (European Food Safety Authority, 2016; Institute of Medicine, 2011). The magnitude of 25(OH)D concentration change over the restoration period was matched between the SSR and ORAL groups, and were higher compared to their respective placebos. In comparison, the typical winter nadir and high prevalence of vitamin D insufficiency were still evident in the placebo groups; 67%, and 83% in SSR-P and ORAL-P respectively. This is the first study that has attempted to compare the change in serum 25(OH)D incurred by an oral dose of vitamin D_3 to a method that replicates 'typical' safe summer sunlight exposure. Specifically, the SSR protocol is in line with current recommendations for safe sunlight exposure (Radiation, 2017), and simulates summer sunlight exposure (at latitudes between 30 and 60°N for most skin types), on several occasions each week, for ~15 minutes wearing t-shirt and shorts without producing sunburn (Webb et al., 2011). Studies that have tried to compare the efficacy of raising serum 25(OH)D concentrations by oral vitamin D_3 and artificial UVB exposures have been limited by a number of factors, including: the use of fluorescent lamps with UV emission removed from that of sunlight, exposure to personalized (MED-related) UV doses rather than a standardised challenge, and near total-body surface exposure, which is unlikely to occur in

everyday life (Armas et al., 2007; Chen et al., 2007; Matsuoka, Wortsman, Haddad, Kolm, & Hollis, 1991). Assessing the equivalent oral vitamin D_3 intake to the SSR regimen is important especially in the context where skin exposure to sunlight containing the requisite UVB is limited, or where the skin is less responsive (Webb et al., 2011). From previous work, it was concluded that a single exposure of the whole body to 1 MED (the minimum amount of sunlight that burns the skin) would typically give an oral equivalent vitamin D dose in the range of 10,000-25,000 IU (Webb et al., 2011); vastly exceeding the purported safe upper limit (SUL) of daily vitamin D_3 consumption. However, based on the findings from the current study it can estimated that a UVR exposure of 1.3 SED, as used by Rhodes and colleagues and the current study, to a white UK population wearing shorts and t shirts provides the oral equivalent of 1,000 IU, well within the SUL of daily vitamin D_3 consumption (Institute of Medicine, 2011).

Over an eight-week maintenance phase, we were able to demonstrate that less frequent SSR exposures (once-a-week) and a reduced oral dose (400 IU/day) maintained elevated 25(OH)D concentrations in our young healthy population. To date, there is little evidence regarding how well serum 25(OH)D is maintained following the SSR regimen developed by Rhodes and colleagues. Apart from the current data, the best observations resolving this issue comes from work conducted by the same research group. Webb & colleagues followed the vitamin D status and UV exposure of a representative white Caucasian Manchester (UK) population throughout a year and identified that individuals who achieved serum 25(OH)D status of ~75 nmol/L at the end of summer, maintained sufficient vitamin D status over the winter (Webb et al., 2010). It should be noted that only 1 participant in the current study achieved the concentration of 75 nmol/L following the simulated summer of thrice-weekly exposures. Therefore it may be concluded that those who have serum 25(OH)D < 75 nmol/L following a period of restoration may benefit from an additional maintenance dose of simulated sunlight or an oral vitamin D equivalent. This conclusion is supported by the visual decline in serum 25(OH)D concentrations among those who were transferred from the active treatments to the respective placebo groups at the start of the 8-week maintenance phase. Although mean serum 25(OH)D did not significantly decline from week 5 to 12, the percentage of individuals displaying sufficient 25(OH)D status declined. Although, this appears to counter evidence from Bogh et al (2012) who have demonstrated a significant decline in serum 25(OH)D concentrations among individuals who do not receive active UVB treatment to maintain summer 25(OH)D status (Bogh, Schmedes, Philipsen, Thieden, & Wulf, 2012). It should be acknowledged that

the decay period was measured over 16 weeks in comparison to our 8 weeks. Albeit in our small sample size, it could be assumed that a similar decline may have been observed if the study went on for a longer duration.

To strengthen our findings, we excluded participants who had been or were planning to travel on holiday to a hot or sunny location, or individuals using supplemental vitamin D_3 before the study. Additionally, the respective courses of SSR or oral vitamin D_3 commenced in February, thereby excluding any influence of ambient UVB. As a result, starting 25(OH)D status was low across all groups, thereby overcoming the limitation of high baseline 25(OH)D as observed in previous supplementation studies (Laaksi et al., 2010; Li-Ng et al., 2009; Murdoch et al., 2012). Indeed, low starting 25(OH)D status is a strong predictor of response to supplementation (Trang et al., 1998). A major strength in the design of this study is in the incorporation of a method to replicate safe summer sunlight exposure using sub-erythemal solar simulated radiation. This experimental protocol incorporated volunteers who wore casual clothing to reveal skin areas more commonly exposed in summer months. Furthermore, the method applies rigorously controlled dosimetry with a lamp UV-emission spectrum closely matched to sunlight, which allows for the influence of UVA and UVB wavelengths on cutaneous vitamin D degradation and therefore enhances the similarity with real life sunlight exposure. One limitation of the current study is the lack of control over the compliance in participants who underwent ORAL treatment. This may have influenced the serum 25(OH)D results of participants in this group, but this is also a reflection of a true clinical scenario. Additionally, the increase in 25(OH)D from week 1 to 12 in the placebo groups and the observation that 25(OH)D concentrations were similar between those in the SSR group compared to SSR-P participants at week 9 show evidence of increases in 25(OH)D separate to the interventions; possibly increasing seasonal sunlight exposure. However, no measure of causal sunlight exposure was attained during the current study.

There were important observations made from the results that informed the design of the study of the following experimental chapter (**Chapter 6**). Firstly, due to convenience, the present study commenced in February but ended in May; therefore increases in ambient sunlight may have contributed to increases in vitamin D status. Future studies should commence in January to limit the influence of natural UV exposure. Secondly, polysulphone film badge dosimeters should be incorporated to estimate the personal sunlight exposure; thereby allowing us to account for increases in skin sunlight exposure to explain increases in vitamin D concentrations

separate to the intervention. Next, the change in 25(OH)D elicited by ORAL supplementation during the four week restoration period was higher, albeit non-significant, in comparison to the SSR regimen (ORAL=38.6 ± 10.8 nmol/L, SSR=29.5 ± 10.3 nmol/L). Therefore it was decided to increase the skin area exposed to the SSR dose from 33%-40%. Lastly, it was decided that participants would receive the reduced SSR and oral vitamin D_3 doses during the 8-week maintenance phase, in order to maintain the typically observed 25(OH)D summer concentrations.

In conclusion, the results from the present study suggest the both 3-times weekly simulated solar radiation (SSR) and daily consumption of an oral vitamin D_3 (1,000 IU/day) supplement over a period of 4 weeks incur a similar change in serum 25(OH)D, and promote vitamin D sufficiency in the majority of young healthy individuals. Secondly, both less frequent SSR exposures (once-a-week) and a reduced oral dose (400 IU/day; March–May) for a further 8 weeks maintained elevated serum 25(OH)D and sufficient vitamin D status in 100% of participants. Given the findings from **Chapter 4** demonstrating that during wintertime, when vitamin D insufficiency is more prevalent (serum 25(OH)D < 50 nmol/L), fewer men and women mounted a secondary anti-HBs response (anti-HBs ≥ 10 mIU/mL), in comparison to those vaccinated during the summer, and the findings of the current study. The next step in the sequence of investigations may seek to investigate whether restoring and maintaining 25(OH)D sufficiency during the winter influences *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccine.

CHAPTER SIX

The influence of vitamin D supplementation by sunlight or matched oral vitamin D_3 doses on secondary anti-HBs response: a randomised placebo controlled trial

6.1 SUMMARY

The current study aimed to determine, in a randomised control trial, whether achieving vitamin D sufficiency, by either safe and practical government recommended summer sunlight or daily oral vitamin D_3 supplementation in winter, influenced the development of secondary anti-HBs response. Following administration of the first hepatitis B vaccine dose, 119 healthy young and healthy men, aged 21 ± 3 years, were randomly allocated to one of four groups. Those allocated to vitamin D groups received an initial 4 week 'restoration' course of either 3x/weekly solar stimulated radiation (SSR) or 1,000 IU/day oral vitamin D_3 capsules (ORAL) designed to achieve vitamin D sufficiency in almost all before the second hepatitis B vaccination. These concentrations were maintained over an eight-week 'maintenance' phase consisting of a course of 1x/weekly SSR exposures or 400 IU/day oral vitamin D_3. Other participants received matched placebos across the two phases (SSR-P, ORAL-P). Serum anti-HBs were assessed at week 1 and after 12 weeks of either vitamin D or placebo supplementations (~8 weeks after the 2^{nd} vaccine dose). Simulated-sunlight and oral vitamin D_3 supplementation were similarly effective to achieve vitamin D sufficiency in almost all (95%); however, vitamin D supplementation after the initial hepatitis B vaccination did not influence secondary anti-HBs response (SSR and ORAL *vs*. SSR-P and ORAL-P; 58% *vs.* 54%, $P > 0.05$). Future studies should seek to raise vitamin D status prior to initial vaccination, using methods that replicate safe, practical, and government recommended summer sunlight and daily oral vitamin D_3 supplementation guidelines.

6.2 INTRODUCTION

The findings presented in **Chapter 4** of this thesis demonstrated a potential role of vitamin D in the development of hepatitis B antibody response in healthy young adults. Indeed, fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations. Furthermore, fewer individuals who received their hepatitis B vaccination during the winter, when vitamin D was lowest, were vaccine responders compared to those vaccinated during the summer and to what is typically expected following 2 out of the three hepatitis B vaccine doses (**Chapter 4**). Then in **Chapter 5** we demonstrated that safe and practical government recommended summer sunlight (SSR) and daily oral vitamin D_3 supplementation regimens administered over 12 weeks achieved and maintained 25(OH)D concentrations at 'typical' summer concentrations and promoted vitamin D sufficiency in almost all participants (~90%). Specifically, simulated sunlight and oral vitamin D_3 supplementation restored 25(OH)D from winter to concentrations typical in summer within 4-weeks and maintained IOM and EFSA vitamin D sufficiency concentrations for a further 8-weeks (serum 25(OH)D $\geq$ 50 nmol/L). The next step in this sequence of studies was to assess whether achieving 25(OH)D sufficiency, by safe and practical government recommended summer sunlight or daily oral vitamin D_3 supplementation during winter influences secondary antibody response to the hepatitis B vaccine.

The current study aimed to further examine the influence of vitamin D on the development of *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccination (anti-HBs). The study was a randomised placebo-control trial conducted during UK winter when vitamin D status was low in most individuals. Vitamin D sufficiency was achieved by 12-weeks vitamin D supplementation, by either simulated sunlight in accordance with recommendations on safe, casual sunlight exposure (Advisory Group on Non-ionising Radiation, 2017), or oral vitamin D_3. A secondary aim was to explore any differential effects on antibody response following hepatitis B vaccination between the two main roots of vitamin D supplementation i.e. oral vitamin D_3 and casual sunlight exposure. Indeed, increasing vitamin D status via UVB exposure of the skin may improve immune function greater than the equivalent oral vitamin D_3 supplementation via nitric oxide mediated immunomodulation (Juzeniene & Moan, 2012), or UV induced enhancement of mood, which is independent of the synthesis of vitamin D. However, controlled UVB exposures have also been shown to have immunosuppressive effects on humoral immune response to hepatitis B vaccine in both mice and humans (Sleijffers et al., 2001, 2002). Whether simulated summer sunlight exposures in

accordance with recommendations for safe, casual sunlight exposure to restore adequate circulating vitamin D in the winter improves immunity and host defence remains largely unknown and requires investigation. We hypothesized that individuals receiving vitamin D supplementation, via controlled SSR exposures or oral vitamin D_3, would have superior secondary anti-HBs response than non-supplemented individuals i.e. placebo groups.

6.3 METHODS

Participant characteristics. Healthy men were enrolled in a double-blind randomised, placebo-controlled trial upon entering the British Army Combat Infantryman's Course, Catterick, UK during January and February of 2016 and 2017, when ambient ultraviolet-B (UVB) was negligible at UK latitudes and vitamin D concentrations were at a seasonal low. Eligible participants were ≥ 17 years of age and cleared Army initial medical assessment; had no history of skin cancer, photosensitivity, or systemic lupus erythematous; and had sun-reactive skin type I–IV (Fitzpatrick, 1988). Participants were excluded for the same reasons described in **Chapter 4**, plus current consumption of vitamin D in dietary supplements; use of a sun bed or travel to a sunny climate 3-months before the study.

Experimental design and procedures. Participants completed the same baseline assessments and the initial hepatitis B vaccination, as described in **Chapter 3 (Chapter 3, section 3.5)**. Additionally skin pigmentation was assessed by measuring lightness (International Commission on Illumination *L**) on a scale from 0 (black) to 100 (white) with a spectrophotometer (CM2500d; Konica Minolta); triplicate readings were taken from the UV exposed inner aspect of the forearm, and UV unexposed inner aspect of the upper arm. Participants were block randomised within their platoons to one of four intervention groups: 1) solar simulated radiation (SSR); 2) solar simulated radiation placebo (SSR-P); 3) oral vitamin D_3 (ORAL); 4) oral placebo (ORAL-P). Block randomisation (using randomizer.org) resulted in an equal distribution of intervention groups within each platoon, and therefore ensured any differences in training conditions between platoons did not influence the study outcomes. An independent researcher completed the randomisation and investigators were blind to the randomisation until after the primary statistical analyses were completed. The interventions began 3 ± 2 days after the initial hepatitis B vaccine dose (**Chapter 3, Figure 3.1**). The intervention strategy for the SSR and ORAL groups was to restore and then maintain vitamin

D sufficiency (serum 25(OH)D ≥ 50 nmol/L) as recommended by Institute of Medicine (IOM) and the European Food Safety Authority (EFSA) (European Food Safety Authority, 2016; Institute of Medicine, 2011). Participants completed a four week restoration phase, necessary because serum 25(OH)D was as its winter nadir, followed by an 8-week maintenance phase. Those assigned to the SSR intervention were exposed three-times-a-week during the restoration phase and once-a-week during the maintenance phases to a controlled constant UVR dose using a whole body irradiation cabinet (Hapro Jade, Kapelle, The Netherlands, as described in **Chapter 3 (Chapter 3, section 3.7.2)**. SSR-P participants received the same exposure on cabinets fitted with UV-blocking films (as described in **Chapter 3, section 3.7.2**). Participants assigned to the ORAL group consumed a daily vitamin D supplement labelled as 1,000 IU vitamin D_3 in the restoration phase and 400 IU D_3 in the maintenance phase (Pure Encapsulations, Sudbury, Massachusetts, USA). ORAL-P participants consumed a daily oral cellulose placebo capsule, identical in size, shape and colour to the vitamin D capsules (Almac Group, County Armagh, UK) (as described in **Chapter 3, section 3.7.1**). Blood samples were obtained at week 1, 5 and 12 for the determination of serum 25(OH)D. Vitamin D from the diet was estimated in week 12 using a food frequency, and solar ultraviolet radiation (UVR) exposure was estimated in weeks 4 and 11 using polysulphone badges (Webb et al., 2010). On completion of the study, participants were asked to guess which intervention they thought they had been receiving.

Week 1 blood collection and hepatitis B vaccination protocol followed procedures from **Chapter 4**. A second blood sample was collected at week 5 for determination of serum 25(OH)D and primary serum anti-HBs response following the restoration phase of the intervention. A third blood sample was collected at week 12, 8 ± 1 week after the second hepatitis B immunisation (3 months after the first hepatitis vaccination) for the determination of secondary serum anti-HBs titres and serum 25(OH)D following the full 12 week study protocol (**Chapter 6, Figure 6.1**). Skin pigmentation was assessed at the end of the 12-week treatment period following the same protocol as described previously.

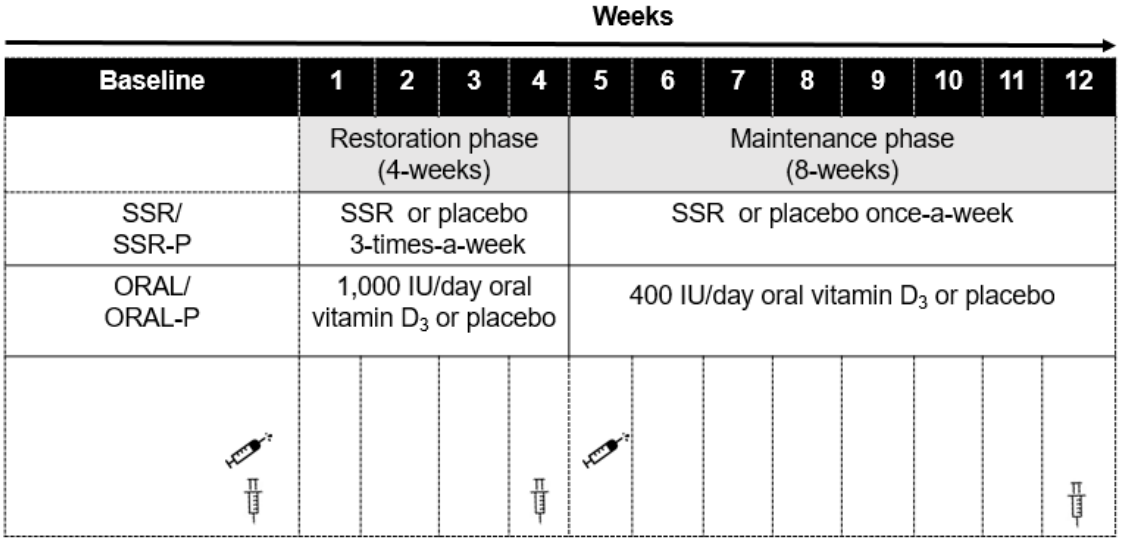

Figure 6.1. Schematic of study procedures. Investigated the influence of a 12-week vitamin D supplementation, by solar simulated radiation (SSR), oral vitamin D_3 (ORAL), or placebo (SSR-P or ORAL-P), that began after the initial hepatitis B vaccination on secondary anti-HBs response. Needle icon indicates when hepatitis B vaccination doses were given. Syringe icon indicates when a blood sample was obtained for serum 25(OH)D and anti-HBs measurement (baseline, week 5 and week 12).

Assessment of dietary vitamin D intake and sunlight exposure. Prior to collecting blood samples at week 12, participants were asked to complete a food frequency questionnaire (FFQ) to assess dietary vitamin D intake during army training. Sunlight exposure was also estimated by polysulphone badge dosimeters during the weeks prior to week 5 and week 12. Participants wore the badges on the outer most piece of clothing in front of their chest (either by their rank slide holder of the outer tunic, or the centre of a t-shirt). One badge was worn from Monday to Friday and a second on Saturday to Sunday. The difference in absorption at 330nm before and after the badge had been worn was used to obtain the standard erythema dose (SED). This dose was then corrected for days participants reported to have worn the badges (SED/day). Alongside polysulphone badges, participants were also asked to complete a daily sun exposure diary, detailing the amount of time spent outside and the clothing worn to determine body surface area exposure (Webb, 2006).

Biochemical analyses. For blood sample preparation, storage and analyses of serum 25(OH)D, 24, $25(OH)_2D$, 1, 25 $(OH)_2D_3$ and anti–HBs titres refer to **Chapter 3, section 3.6**. Intra and

inter assay CVs for the assessment of anti-HBs using ELISA (DiaSorin, Saluggia, Italy) were 5.9% and 22.3% respectively.

Statistical analysis. The results from **Chapter 5** confirmed that both SSR and ORAL treatment protocols, as used in the described study, were successful in raising serum 25(OH)D by ~ 30 nmol/L, and promoted vitamin D sufficiency (serum 25(OH)D ≥ 50nmol/L) in ≥ 90% of healthy young individuals. Therefore, the main analyses compared vitamin D supplementation (SSR and ORAL combined together) and placebo groups i.e. SSR and ORAL *vs.* SSR-P and ORAL-P. The estimation of sample size was based on the anticipated 20% difference in percentage vaccine responder rates chronic kidney disease patients, with low *vs.* high vitamin D status obtained from Zitt and colleagues (Zitt et al., 2012) (45% vs. 65% respectively). An equally divided sample of 152 (n = 76 in each group) was estimated for the detection of a 20% difference in vaccine response, with a type 1 error (one tailed) of 5% and a power of 80%, based on a sample size estimate for comparing the proportions in two independent groups. Primary analysis consisted of a chi-square test to compare the percentage of secondary anti-HBs responders (anti-HBs ≥ 10mIU/mL) after vitamin D supplementation (SSR and ORAL combined together) and placebo groups (SSR-P and ORAL-P combined together). Secondary analysis consisted of comparing the percentage of secondary anti-HBs responders among the individual vitamin D supplementation groups after vitamin D supplementation versus their respective placebo groups (SSR *vs.* SSR-P, ORAL *vs.* ORAL-P). Furthermore, similar analyses were also conducted excluding those who were vitamin D sufficient at baseline, and in those who achieved optimal vitamin D status (serum 25(OH)D ≥ 75 nmol/L) after the initial 4 week restoration period. Demographic, anthropometric, lifestyle behaviours were compared between vitamin D and placebo groups by independent samples *t*-test and Mann-Whitney *U*-tests for normally and non-normally distributed data, respectively. Vitamin D metabolites, serum 25(OH)D, 1, 25$(OH)_2$D and 24, 25(OH)D were compared between vitamin D and placebo groups using a 2 x 3 repeated measures ANOVA. Where significant interaction or main effects were observed, post-hoc pairwise comparisons were conducted using Bonferroni adjusted *t*-tests. Chi-square tests were conducted to compare the percentage of participants displaying sufficient vitamin D (serum 25(OH)D ≥ 50 nmol/L) at week 1, week 5 and week 12 between vitamin D and placebo groups.

6.4 RESULTS

Participants flow and characteristics. Two hundred and thirty one men were assigned to the interventions in January and February of 2016 and 2017. Participant flow, drop out and exclusion before biochemical and statistical analysis is summarised in **Figure 6.2**. A summary of the baseline anthropometry, demographics and lifestyle behaviours are presented for the final n = 119 in **table 6.1**. At baseline, there were no differences between the vitamin D and placebo supplementation group's demographic and anthropometric characteristics, lifestyle behaviours, sleep or mood (Table 6.1). During the 12-week intervention, daily sunlight exposure was low, as is expected considering the latitude and time of year (Macdonald, 2013). Both estimated sunlight exposure (0.22 ± 0.33 SED/day; $P > 0.05$) and dietary vitamin D intake were not different between groups (112 ± 84 IU/day, $P > 0.05$). Participants were sufficiently blinded to the intervention since only 35% correctly guessed their allocated group, 30% were incorrect, 35% said they did not know whether they had received an active or placebo intervention.

Safe simulated sunlight and oral vitamin D_3 restored vitamin D sufficiency in almost all. At baseline, three quarters (75%) of volunteers were vitamin D insufficient (25(OH)D < 50 nmol/L). The supplementation strategies were successful in achieving vitamin D sufficiency and maintaining serum 25(OH)D concentrations so that at week 5 and 12 serum 25(OH)D concentrations in the combined vitamin D supplementation group (SSR and ORAL) were higher than the combined placebo groups ($P < 0.05$; **Figure 6.3A**). Indeed, by week 5 almost all participants in the SSR and ORAL groups were vitamin D sufficient (95%: 25(OH)D ≥ 50 nmol/L; **Figure 6.3B**). Serum 1, $25(OH)_2D$ was similar in all groups at baseline ($P > 0.05$) and increased with supplementation (**Figure 6.3C,** $P < 0.05$), with SSR and ORAL groups greater than the placebo groups at week 5 ($P < 0.05$).

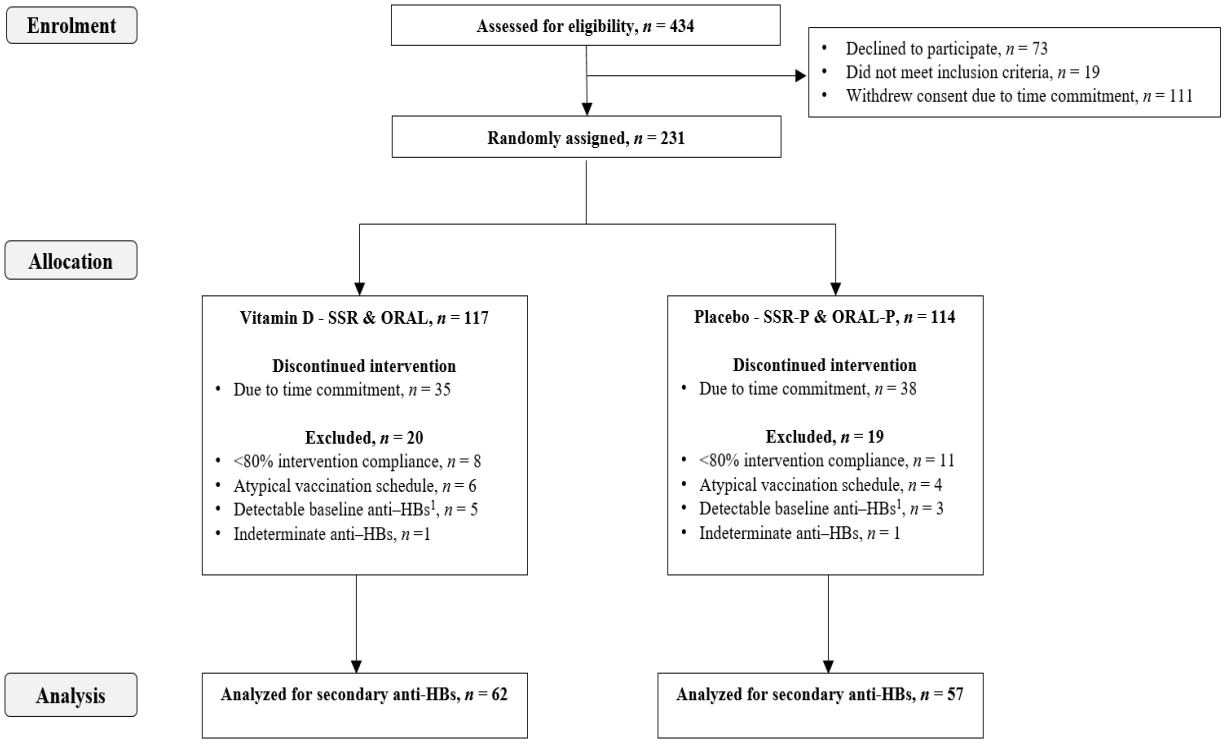

Figure 6.2. Flow diagram, indicating the number of participants assessed, recruited and analysed as part of the study. [1]Anti-HBs; antibodies against hepatitis B antigen. Vitamin D = SSR; solar simulated radiation, ORAL; oral vitamin D_3. Placebo = SSR-P; solar simulated radiation placebo.

There was no difference between groups at week 12 ($P > 0.05$) because 1,25$(OH)_2$D increased from week 5 to 12 in placebo groups ($P < 0.05$). The inactivated metabolite 24, 25(OH)D, showed comparable values between vitamin D supplemented groups (SSR and ORAL) and the placebo groups (SSR-P and ORAL-P). By week 12 both the vitamin D supplemented and placebo groups showed increases in the metabolite, ($P < 0.05$; **Table 6.2**). An interaction effect was also detected as the vitamin D supplemented groups displayed markedly higher peak 24, 25(OH)D concentrations at week 12.

Other studies have also examined the ratios of 25(OH)D and 1, 25$(OH)_2$D to 24, 25(OH)D. It has been suggested that the ratio of 25(OH)D to 24, 25(OH)D is predictive of the 25(OH)D response to supplementation (Kaufmann et al., 2014; Ketha, Kumar, & Singh, 2016; Molin et

al., 2015). In the case of the current study we calculated ratio data for the relationships between 25(OH)D and 1, $25(OH)_2D$ and 1, $25(OH)_2D$ to 24, 25(OH)D. Ratios for the vitamin D supplemented groups (SSR and ORAL) decreased by week 12 (P <0.05; **Table 6.2**). Comparatively, 25(OH)D:24, 25(OH)D was maintained in the placebo groups, whilst 1, $25(OH)_2D$:24, 25(OH)D declined by week 12.

Vitamin D supplementation after initial hepatitis B vaccination effect on secondary anti-HBs. Overall, 56% of men responded to the second hepatitis B vaccination (anti-HBs ≥ 10 mIU/mL). Vitamin D supplementation after the initial vaccination did not influence secondary anti-HBs response, as the percentage of secondary anti-HBs responders was similar among the vitamin D and placebo groups (**Figure 6.3D**, P > 0.05). Further, there was no difference in secondary anti-HBs response after vitamin D supplementation when excluding those that were vitamin D sufficient at baseline (P > 0.05, data not shown). Secondary analysis of SSR or ORAL supplementation versus their respective placebo groups (SSR-P & ORAL-P, respectively) also revealed no effect of either vitamin D supplementation method on secondary anti-HBs response (P > 0.05). Additionally, analyses restricted to participants with vitamin D insufficiency before vitamin D_3 supplementation (n = 89; SSR & ORAL = 41, SSR-P & ORAL-P = 48), revealed similar findings (% anti-HBs ≥ 10 mIU/mL, SSR & ORAL *vs.* SSR-P & ORAL-P; 59% *vs.* 60%). In an effort to further explore the clinical significance of the 50 nmol/L serum 25(OH)D cut off for *in vivo* immunity, additional chi-square analyses were conducted, irrespective of treatment, which assessed whether individuals who were vitamin D sufficient following initial vaccination subsequently mounted stronger hepatitis B vaccine responses. Similar to the main statistical analyses, secondary hepatitis B vaccination response was similar between participants who presented as vitamin D sufficient *vs.* insufficient both following the restoration phase (Week 5, % anti-HBs ≥ 10 mIU/mL, < 50 nmol/L *vs.* ≥ 50 nmol/L; 56 % *vs.* 56%) and following the full supplementation protocol (Week 12; % anti-HBs ≥ 10 mIU/mL, < 50 nmol/L *vs.* ≥ 50 nmol/L; 57% *vs.* 56%).

Table 6.1. Participant demographics, anthropometrics, and lifestyle characteristics for SSR and ORAL *vs.* SSR-P and ORAL-P.

	Vitamin D (SSR and ORAL combined) n= 62	Placebo (SSR-P and ORAL-P combined) n = 57
Demographics		
Age (years)	21.2 ± 2.9	21.5 ± 3.2
Ethnicity (Caucasian) [*n* (%)]	61 (98)	57 (100)
Anthropometrics		
Height (cm)	177 ± 6	177 ± 6
Body mass (kg)	76 ± 12	77 ± 10
BMI (kg/m^2)	24.2 ± 3.2	24.6 ± 2.7
Lifestyle behaviours		
Alcohol user [*n* (%)]	49 (79)	45 (79)
Smoker [*n* (%)]	34 (55)	27 (47)
Week 1 Skin pigmentation (*L**)		
Inner forearm – UV exposed	68 ± 2	68 ± 2
Upper arm – UV unexposed	70 ± 2	70 ± 2
Sleep night before initial vaccination		
Duration (h)	6.0 ± 1.3	5.9 ± 1.6
Quality (very poor =1 to very good =4)	3 ± 1	3 ± 1
Mood before initial vaccination		
Depression	0.7 ± 1.6	0.8 ± 1.9
Fatigue	4 ± 3	4 ± 3
Tension	3 ± 3	3 ± 3

Values presented as mean ± SD unless otherwise stated. There were no significant differences between vitamin D and placebo supplemented groups in demographics, anthropometrics, lifestyle behaviours, sleep or mood at baseline ($P > 0.05$).

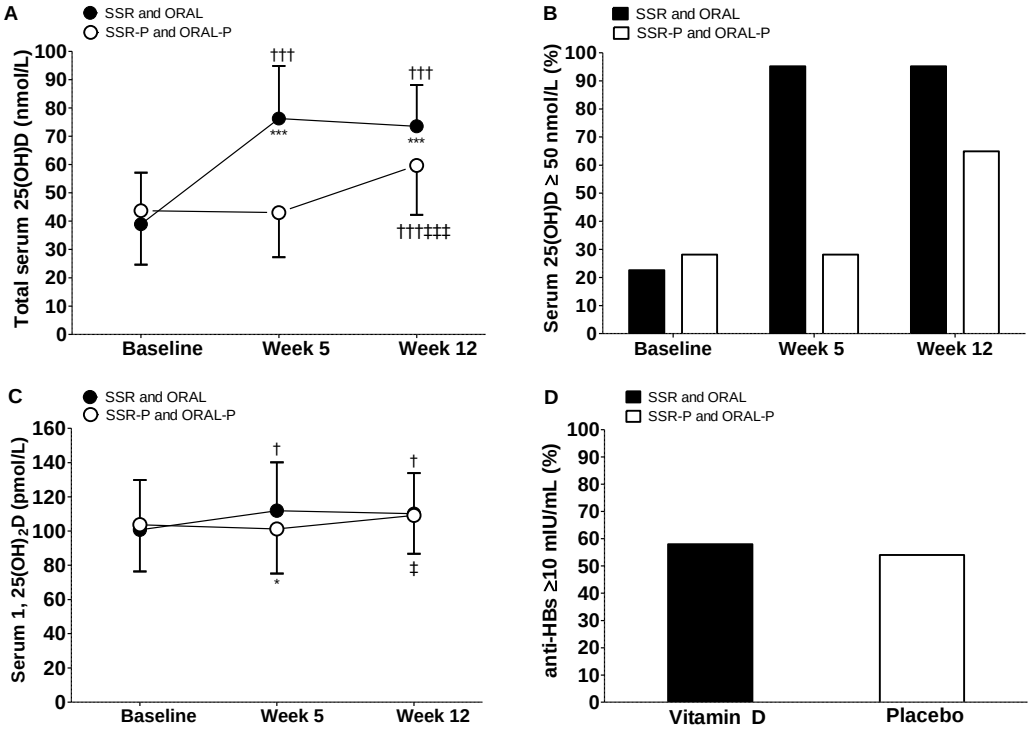

Figure 6.3. Serum 25(OH)D (panel A), percentage of participants categorised as vitamin D sufficient (25(OH)D ≥ 50 nmol/L, panel B), serum 1,25$(OH)_2$D (panel C) and percentage of participants categorized as anti-HBs responders (anti-HBs ≥ 10 mIU/mL, panel D) following 12-weeks of vitamin D supplementation that began after the initial hepatitis B vaccination. Panels A, B, C, D show combined vitamin D supplementation (SSR and ORAL) *vs.* combined placebo (SSR-P and ORAL-P) groups. † $P < 0.05$ and ††† $P < 0.001$, greater than week 1. ‡ $P < 0.05$ and ‡‡‡ $P < 0.001$, greater than week 5. * $P < 0.05$ and *** $P < 0.001$, greater than placebo. Data are mean ± SD (panels A and C) and vertical bars represent percentages (panel B and D).

Table 6.2. Serum responses of serum vitamin D metabolites and vitamin D metabolites expressed as ratio at all test time points in both the SSR and SSR – P treatment groups. Data are mean ± SD; a *P* 0.05 greater than week 1, b *P* 0.05 greater than week 5, c *P* 0.05 greater than week 12. † *P* < 0.05 and ††† *P* < 0.001, greater than placebo. * *P* 0.05, ** *P* 0.01, *** *P* 0.001 greater than active vitamin D treatment.

A.

Treatment group	SSR (n = 30)			SSR – P (n = 28)		
Measure	Week 1	Week 5	Week 12	Week 1	Week 5	Week 12
Serum 24, 25(OH)D (nmol/L)	2.5 ± 1.8	5.3 ± 1.6^{a†††}	5.7 ± 1.5^{a†††}	2.3 ± 1.5	2.2 ± 1.3	3.4 ± 1.5^{a,b}
Ratio 25(OH)D:24, 25(OH)D	19.9 ± 6.9	16.2 ± 3.8^{a}	14.5 ± 3.0^{a,b}	20.3 ± 8.6	20.5 ± 5.2***	18.4 ± 3.6***
Ratio 1, $25(OH)_2D$:24, 25(OH)D	64.5 ± 47. 1^{b,c}	24.2 ± 10.5	21.7 ± 7.3	61.2 ± 54.4^{c}	53.6 ± 30.6^{c***}	34.7 ± 12.7***

B.

Treatment group	Oral (n = 32)			Oral - P (n = 28)		
Measure	Week 1	Week 5	Week 12	Week 1	Week 5	Week 12
Serum 24, 25(OH)D (nmol/L)	2.3 ± 1.5	4.7 ± 1.6^{a†††}	4.8 ± 1.2^{a†††}	3.2 ± 1.8^{b,c*}	2.7 ± 1.5^{c}	3.5 ± 1.3
Ratio 25(OH)D:24, 25(OH)D	18.7 ± 5.9 ^{b,c*}	15.7 ± 3.2	14.8 ± 3.0	16.1 ± 4.1	18.0 ± 5.8^{a*}	17.8 ± 4.0**
Ratio 1, $25(OH)_2D$:24, 25(OH)D	67.3 ± 60.4^{b,c}	26.0 ± 11.0	23.3 ± 7.0	46.1 ± 29.3	51.1 ± 35.1^{c***}	38.3 ± 24.6**

C.

Treatment group	Vitamin D (n = 62)			Placebo (n = 57)		
Measure	Week 1	Week 5	Week 12	Week 1	Week 5	Week 12
Serum 24, 25(OH)D (nmol/L)	2.4 ± 1.7	5.0 ± 1.6^{a†††}	5.2 ± 1.4^{a†††}	2.8 ± 1.7	2.5 ± 1.4	3.5 ± 1.4^{a,b}
Ratio 25(OH)D:24, 25(OH)D	19.3 ± 6.4^{b,c}	16.0 ± 3.5^{c}	14.7 ± 3.0	18.0 ± 7.0	19.3 ± 5.4***	18.0 ± 3.8***
Ratio 1, $25(OH)_2D$:24, 25(OH)D	65.9 ± 53.9^{b,c}	25.1 ± 10.7	22.5 ± 7.1	53.2 ± 44.3^{c}	52.4 ± 32.3^{c***}	36.3 ± 19.8***

6.5 DISCUSSION

In a randomised, placebo-controlled trial, the current study aimed to examine the influence of achieving IOM and EFSA recommended vitamin D sufficiency (serum 25(OH)D ≥ 50 nmol/L) on the development of *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccination (anti-HBs). The study was completed during the winter where vitamin D concentrations were anticipated to be low. Indeed, 75% of the total cohort presented with vitamin D insufficiency (serum 25(OH)D < 50 nmol/L) at baseline. The initial four weeks of the vitamin D supplementation regimens used in the current study successfully resolved vitamin D insufficiency in 95% and increased serum 25(OH)D concentration by ~ 37 nmol/L among participants. Following this, a further 8 week maintenance phase of once weekly SSR and 400 IU/day oral vitamin D_3 maintained vitamin D sufficiency and elevated serum 25(OH)D concentrations. Conversely, 35% of individuals who were allocated to placebo treatments remained at potential risk of poor health with a mean total 25(OH)D concentration < 50 nmol/L at week 12. However, vitamin D supplementation did not influence secondary anti-HBs response (SSR and ORAL *vs.* SSR-P and ORAL-P; 58% *vs.* 54%). These findings were replicated in additional analyses; firstly restricted to participants with vitamin D insufficiency prior to vitamin D_3 supplementation, and secondly in those who achieved the proposed optimal serum 25(OH)D ≥ 75 nmol/L. Although, no influence of increasing vitamin D status by safe, simulated sunlight or oral vitamin D_3 was observed on hepatitis B vaccine response, this result confounds the immunosuppressive effects of controlled UVB exposure on humoral immune response to hepatitis B vaccine and the contact hypersensitive response to the novel allergen DPCP in mice and human models respectively (Sleijffers et al., 2001, 2002). The applicability of these studies can be questioned due to the use of mouse models, and the UVB regimens involve total body exposure to personalized MED doses, which are of questionable relevance to real life sun exposures. Comparatively we adopted an ecologically valid method that replicated typical safe summer sunlight exposure (at latitudes 30-60 °N, for most skin types) (Farrar et al., 2011). Therefore, the current study demonstrates that UVB derived vitamin D, when administered within government guided recommendations, does not confer immunosuppression as previously shown.

Whether vitamin D is important for initial capturing and processing of an antigen at the site of vaccination or the subsequent downstream effects leading to the development of immune

memory is not well understood (Lang & Aspinall, 2015). In the current study we were only able to manipulate serum 25(OH)D following the initial hepatitis B vaccination. Therefore, participants were still low in vitamin D at the time of the initial vaccine challenge. In the context of stress immunology, the current findings agree with human and animal studies that show stress modulates the development of new immunity when it occurs in close proximity to the antigen exposure. Animal literature suggests that stress must occur immediately before the first antigen exposure to alter *in vivo* immune induction (Fleshner et al., 1992; Okimura et al., 1986). Stress occurring after this period has been shown to have no effect (Fleshner et al., 1992; Wood, Karol, Kusnecov, & Rabin, 1993). Although much less studied, investigations in humans show similar findings to animal studies where stress in close proximity to the first immune challenge has an immune modulatory effect, but stress close to the recall has a greatly reduced (Harper Smith et al., 2011) or no effect (Smith et al., 2004; Zachariae, Jorgensen, Christensen, & Bjerring, 1997). When applied to the current context, it is conceivable that changing vitamin D status following initial vaccination may have little effect and that vitamin D status at the time of initial vaccination may be of greater importance. Considering that the majority of participants were vitamin D insufficient at baseline and displayed the typical winter nadir in serum 25(OH)D, it is of little surprise that similar secondary anti-HBs responses were observed between vitamin D supplemented (SSR and ORAL) and placebo groups (SSR-P and ORAL-P). In contrast, we have shown that a greater percentage of individuals with higher vitamin D status (25(OH)D and 1, 25(OH2D) at the time of initial vaccination were secondary anti-HBs responders compared to those with lower vitamin D status at the time of initial vaccination (**Chapter 4; Figure 4.4**).

In ~60% of our participants vitamin D supplementation achieved the proposed optimal serum 25(OH)D ≥ 75 nmol/L (He, Aw Yong, et al., 2016) but this did not lead to any beneficial effect on secondary anti-HBs response. Whether larger vitamin D doses achieving greater than normal seasonal changes in serum 25(OH)D (e.g. > 100 nmol/L), would have beneficial effects on *in vivo* immunity remains unclear. Although this may appear to the logical next step in supplementations studies, larger vitamin D doses may be ineffective because they will further increase 24,25$(OH)_2$D (Owens et al., 2017), which might impair VDR–1,25$(OH)_2$D mediated adaptations beneficial for *in vivo* immune responses, like antibody development. Additionally, higher doses of SSR and oral vitamin D_3 risk skin damage and vitamin D toxicity, respectively (Advisory Group on Non-ionising Radiation, 2017; Institute of Medicine, 2011).It is important

to note that the increase observed in 24,25$(OH)_2$D in this study was far lower than shown in one recent publication (Owens et al., 2017). These differences are likely attributable to the employment of very different vitamin D doses in excess of the tolerable upper intakes (4,000 IU/day). We adapted a method to replicate safe summer sunlight exposure using sub erythemal solar simulated radiation in a laboratory-based irradiation cabinet (Rhodes et al., 2010). Importantly, this methods simulates frequent (several times per week), brief (~15 minutes), summer sunlight exposure (wearing a t-shirt and shorts) at latitudes between 30 and 60°N, without causing erythema (Rhodes et al., 2010). The oral vitamin D_3 doses of 1,000 IU/day and 400 IU/day were selected as it was shown to elicit the same magnitude of change and subsequent maintenance of serum 25(OH)D as the described SSR protocol (**Chapter 5**). Significantly, these doses are regarded to be well within the tolerable upper limit of daily vitamin D_3 consumption of 4,000 IU/day as suggested by the US IoM (Institute of Medicine, 2011).

In conclusion, despite increasing vitamin D status and correcting vitamin D sufficiency in ≥ 90% of participants allocated to experimental treatments, no subsequent influence was observed on the development of *in vivo* immune response. However, given that we were only able to manipulate serum 25(OH)D following the initial hepatitis B vaccination, participants were still low in vitamin D at the time of the initial challenge. It is recommended that future studies should seek to raise vitamin D status prior to initial vaccination using methods that replicate safe and practical government recommended summer sunlight and daily oral vitamin D supplementation guidelines.

CHAPTER SEVEN

GENERAL DISCUSSION

This thesis set out to undertake a series of investigations assessing the influence of vitamin D on *in vivo* immunity, as assessed by the secondary antibody response to the hepatitis B vaccine. In **Chapter 4**, a prospective cohort study of 447 young healthy persons, completed across all seasons, we showed that fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations (**Chapter 4, Figure 4.4**). Of particular clinical interest is that vaccine responses in vitamin D insufficient men were lower than typically expected after two hepatitis B vaccine doses. This direct association between vitamin D and successful hepatitis B vaccination might explain the seasonal variation in hepatitis B vaccine response also observed in (**Chapter 4, Figure 4.2D)**. Specifically, fewer men and women who received their initial hepatitis B vaccination during the winter mounted a response to the hepatitis B vaccination in comparison to men and women vaccinated during summer months. These associations were stronger in men compared to women, likely due to a greater prevalence of vitamin D insufficiency in men compared to women (serum 25(OH)D ≥ 50 nmol/L; men *vs.* women, 49% *vs.* 70%, **Chapter 4, Figure 4.2**). Then in **Chapter 5**, we successfully demonstrated that both simulated safe sunlight exposure and matched government guided safe oral vitamin D_3 doses administered over twelve weeks could resolve winter nadirs in vitamin D concentrations; by restoring and maintaining vitamin D sufficiency in ≥ 90% of participants. Lastly, in **Chapter 6**, a double blind randomised controlled trial, vitamin D supplementation by safe, simulated sunlight or oral vitamin D_3 after the initial hepatitis B vaccination did not subsequently influence anti-HBs response status (anti-HBs titres ≥10 mIU/mL) (**Chapter 6. Figure 6.3D**).

7.1 Prevalence of vitamin D insufficiency and deficiency in young healthy adults

Across all three experimental chapters (**Chapters 4-6**) > 75% individuals who had baseline vitamin D status assessed during winter presented with vitamin D insufficiency (serum 25(OH)D < 50 nmol/L). Additionally, 33% of individuals presented with deficient vitamin D concentrations (serum 25(OH)D < 30 nmol/L) during the winter. As logic dictates, vitamin D status is significantly compromised during winter months at latitudes above 35°, when skin sunlight UVB exposure and endogenous synthesis is low. Specifically, over the 6 month winter

and spring period (October – April) all of Scandinavia, most of western Europe (including 90% of the UK) and 50% of North America lies above the latitude that permits exposure to the UVB wavelength necessary for vitamin D synthesis (Pearce & Cheetham, 2010). In the United Kingdom alone, > 50% of the adult population display insufficient vitamin D status and >16% are deficient during the winter and spring (He, Aw Yong, et al., 2016). Specific classifications for vitamin D status in relation to immunity do not exist. However, it is generally accepted that young populations may be at risk of poor bone health, owing to serum 25(OH)D concentrations < 50 nmol/L (Holick, 2004). Therefore, these data suggest that insufficient and even more severe deficient vitamin D concentrations are common among otherwise healthy young adults residing in the United Kingdom.

7. 2. Important influencers for hepatitis B vaccination response

There are several factors known to contribute to an inadequate hepatitis B vaccine response in otherwise healthy young adults, including; male sex, BMI ≥ 25, smoking, poor sleep and psychological stress (Averhoff et al., 1998; Glaser et al., 1992; Prather et al., 2012; Roome et al., 1993; Westmoreland et al., 1990; Wood, MacDonald, et al., 1993; Yang et al., 2016). A particular strength of the studies presented in **Chapter 4** and **6** was the relative homogeneity in these factors in our study populations (Table 4.1 and 6.1). This might be best explained by acknowledging the characteristics of the study population's i.e. military personnel. From an experimental perspective British Military recruits follow a rigid training schedule that likely contributes to homogenous data i.e. self-reported hours of sleep. However, statistically, this does not elicit the spread of data required to assess how such factors may impact on *in vivo* immune response. In addition, British military recruits are medically screened for sleep and psychiatric disorders that are incompatible with military training. Both factors may also impact on self-reported negative mood states (depression, fatigue and tension) as well as sleep quantity and quality. Our population were deemed both physiologically and psychologically healthy to complete army training and therefore unlikely to report compromised sleep or negative mood states that may be indicative of high levels of stress. It should also be appreciated from a methodological perspective that sleep duration was self-reported and recalled retrospectively. Although reporting bias is less likely in healthy participants than those with sleep or psychiatric disorders (Cohen, Doyle, Alper, Janicki-Deverts, & Turner, 2009). Alternatively, actigraphy would provide more accurate characterisation of sleep duration but may present practical and cost challenges in a large sample size as in **Chapter 4.**

However, in agreement with previous literature, we observed that women responded better than men to the hepatitis B vaccination. While specific details about the mechanisms mediating how men and women differ in response to vaccination are lacking (Klein et al., 2015). It has been previously been suggested that superior response to vaccination among women may be owing to the opposite effects of sex hormones, with oestrogen implicated in promoting antibody production and androgens exhibiting inhibitory effects on antibody production by B cells (Furman et al., 2014; Lu et al., 2002). However, greater vaccination responses observed in pre-pubertal girls compared to boys, and post-menopausal women compared to elderly men suggest more inherent genetic and microbiota differences between women and men may better explain the sex based differences in vaccination response (Klein et al., 2015).

We add to the current literature by demonstrating how vitamin D may play an important role in the development of antibody response following hepatitis B vaccination in young and otherwise healthy individuals. Indeed, fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations, compared to those with vitamin D sufficiency and higher $1,25(OH)_2D$ concentrations. Further, both women and men who were vaccinated during the winter, when serum 25(OH)D and $1,25(OH)_2D$ were lowest, had a response rate lower than summer and compared to what is normally expected following 2 out of the 3 hepatitis B vaccine doses (>50%) (Joines et al., 2001). The direct association of vitamin D, and the seasonal variation in the both the major circulating (25(OH)D) and the more novel biologically active, 1, $25(OH)_2D$, provides evidence that vitamin D is an important influencing factor for the development of the hepatitis B vaccination responses. The potential role of vitamin D may be mediated via its direct interaction with antigen presenting cells and more particularly with dendritic cells. These roles may include dendritic cell activation, via TLRs by vaccine antigen, and intracellular upregulation of CYP27B1 with the induction of cytokine production and T cell proliferation (Lang & Aspinall, 2015). Furthermore, animal studies have shown that $1,25(OH)_2D$ induced dendritic cell migration to stimulate T and B-cell antibody response to diphtheria vaccination (Enioutina et al., 2008, 2009). Although, no influence of increasing vitamin D status by safe, simulated sunlight or oral vitamin D_3 was observed on hepatitis B vaccine response, this result confounds the immunosuppressive effects of controlled UVB exposure on humoral immune response to hepatitis B vaccine and the contact hypersensitive response to the novel allergen DPCP in mice and human models respectively (Sleijffers et al., 2001, 2002). The applicability

of these studies can be questioned due to the use of mouse models, and the UVB regimens involve total body exposure to personalized MED doses, which are of questionable relevance to real life sun exposures. Comparatively we adopted an ecologically valid method that replicated typical safe summer sunlight exposure (at latitudes 30-60 °N, for most skin types) (Farrar et al., 2011). Therefore, the findings presented in **Chapter 6** demonstrate that UVB derived vitamin D, when administered within government guided recommendations, does not confer immunosuppression as previously shown.

7.3. Vitamin D status and proximity to initial hepatitis B vaccination

Chapter 6 did not demonstrate an influence of vitamin D on secondary hepatitis B vaccine response. The findings, however, are in keeping with the limited research findings assessing the influence of vitamin D supplementation on *in vivo* immunity in humans, specifically *in vivo* responses following vaccination. For instance, a placebo blinded trial involving the intramuscular administration of 1.0 μg 1, 25 $(OH)_2D$ at the site adjacent to the site of vaccination with influenza vaccine did not find any significant differences in post vaccination HAI response between treatment and placebo groups (Kriesel & Spruance, 1999). However, it should be noted that the experimenters failed to measure either baseline or post calcitriol serum vitamin D status. Secondly, significant pre vaccination HAI titres were measured in nearly all participants. This indicates that the participants had considerable immunity to the three influenza strains before their vaccination. Since a clear inverse relationship exists between pre-immunisation serum HAI titres and antibody response after vaccination, this could have masked potential vaccine enhancing effects of 1, $25(OH)_2D$. In another example, fifty-nine children out of a cohort of 116 children (mean age; 3 ± 1 years) were supplemented with 1,000 IU/day of vitamin D_3 for 4 months, whilst the remaining, 57 received a placebo. As before, this study revealed no significant difference in post vaccination immune response between the treatment and the placebo group (Principi et al., 2013). The similarity between the experimental design used in **Chapter 6** of this thesis and the aforementioned study should be noted, in that both manipulated vitamin D status following the initial vaccine challenge. In both instances, baseline vitamin D concentrations were similar between the vitamin D supplemented groups and placebo groups at the time of receiving the first vaccine challenge. These findings might be best explained in the context of stress immunology. Animal suggests that the stress must occur immediately before the first antigen exposure to alter *in vivo* immune (Fleshner et al.,

1992; Okimura et al., 1986). Comparatively, stress occurring after this period has been shown to have no effect (Fleshner et al., 1992) (Wood, Karol, et al., 1993).

Although much less studied, investigations in humans show similar findings to animal studies where stress in close proximity to the first immune challenge has an immune modulatory effect, but stress close to the recall has a greatly reduced (Harper Smith et al., 2011) or no effect (Smith et al., 2004) (Zachariae et al., 1997). In the context of the study outlined in **Chapter 6** and that of Principi et al, it is conceivable that changing vitamin D status following initial vaccination may have little effect and that vitamin D status at the time of initial vaccination may be of greater importance. Considering that the majority of participants were vitamin D insufficient at baseline and displayed similar concentrations of serum 25(OH)D, it is perhaps of little surprise that similar secondary anti-HBs responses were observed between the vitamin D supplemented groups and the placebo groups in both studies. One previous study observed higher IgG responses in response to tetanus toxoid vaccination following 9 weeks of vitamin D supplementation, compared to a placebo group (Heine et al., 2011). The findings presented in **Chapter 4** supports this, as we showed that a greater percentage of individuals who displayed higher vitamin D status (25(OH)D & $1,25(OH)_2D$) at the time of initial vaccination, were secondary anti-HBs responders compared to those with lower vitamin D status (**Chapter 4, Figure 4.4**). As such, vitamin D may represent an easily modifiable factor to improve hepatitis B vaccine uptake, and therefore disease prevention, unlike other factors such as age and sex.

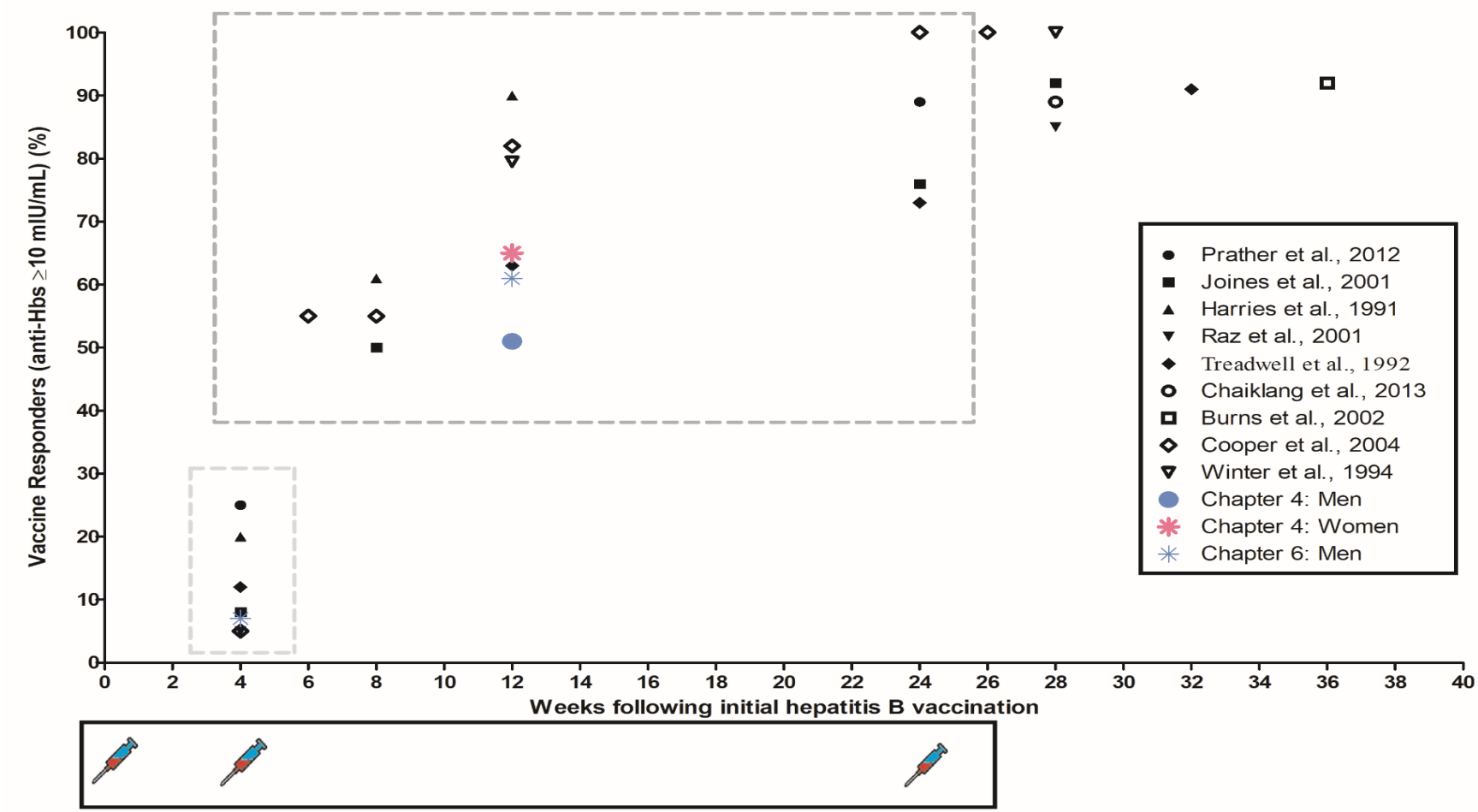

Figure 7.1. Typical anti-HB percentage response following each hepatitis B vaccine dose (20µg recombinant hepatitis B vaccine) including results presented in **Chapter 4** and **6**.

7.4 Supplementation to achieve vitamin D sufficiency during the winter

Although specific classifications for vitamin D status in relation to immunity do not exists, it is generally accepted that young populations may be at risk of poor bone and overall health, owing to serum 25(OH)D concentrations < 50 nmol/L (Holick, 2004). Findings in **Chapter 5** and **6** demonstrate that supplementation methods of thrice weekly exposure and 1,000 IU/day of vitamin D_3 for four weeks achieved EFSA and IOM defined vitamin D sufficiency in almost all participants (European Food Safety Authority, 2016; Institute of Medicine, 2011). The magnitude of 25(OH)D concentration change over the restoration period was matched between the SSR and ORAL groups. In comparison, the typical winter nadir in vitamin D concentrations and high prevalence of vitamin D insufficiency were still evident in the placebo groups; (SSR-P and ORAL-P, **Chapter 5**; 67%, and 83%, **Chapter 6**; 75% and 69%). These data, specifically in **Chapters 5** and **6**, are the first to compare the change in serum 25(OH)D incurred by an oral dose of vitamin D_3 to a method that replicates 'typical' safe summer sunlight exposure. The adopted SSR protocol is in line with current recommendations for safe sunlight exposure (Radiation, 2017), and simulates summer sunlight exposure (at latitudes between 30 and 60°N for most skin types), on several occasions each week, for ~15 minutes wearing t-shirt and shorts without producing sunburn (Webb et al., 2011). Studies that have tried to compare the efficacy of raising serum 25(OH)D concentrations by oral vitamin D_3 and artificial UVB exposures have been limited by; the use of fluorescent lamps with UV emission removed from that of sunlight, exposure to personalized (MED-related) UV doses rather than a standardised challenge, and near total-body surface exposure, which is unlikely to occur in everyday life (Armas et al., 2007; Chen et al., 2007; Matsuoka et al., 1991).

Prior to the results presented in **Chapters 5** and **6** of these thesis, no studies to date directly explored how well 25(OH)D is maintained following the SSR regimen. The best observations addressing this comes from work conducted by Webb & colleagues (Webb et al., 2010). Vitamin D status and UV exposure of a representative white Caucasian Manchester (UK) population were monitored throughout a year. It was observed that individuals who achieved 25(OH)D status of ~75 nmol/L at the end of summer, maintained sufficient vitamin D status over the winter (Webb et al., 2010). It should be noted that some, but not all, attain the concentration of 75 nmol/L following the thrice-weekly SSR protocol for four weeks in both **Chapter 5** and **6**. Albeit in a sub-sample, we did observe a visual decline in serum 25(OH)D concentrations among those who were transferred from vitamin D supplemented groups (SSR

& ORAL) to the respective placebo in **Chapter 5** over an 8 week period following the initial four week restoration phase. In contrast, participants who went onto receive less frequent SSR exposure (once-a-week) and a reduced oral dose (400 IU/day) over an eight week maintenance phase, maintained elevated 25(OH)D concentrations. These latter findings were replicated in our larger male sample in **Chapter 6**. Importantly, both supplementation methods maintained 25(OH)D concentration to a similar degree. Assessing the equivalent oral vitamin D_3 intake to the SSR regimen is important especially in the context where skin exposure to sunlight containing the requisite UVB is limited, or where the skin is less responsive (Webb et al., 2011). From previous work, it was concluded that a single exposure of the whole body to 1 MED (the minimum amount of sunlight that burns the skin) would typically give an oral equivalent vitamin D dose in the range of 10,000-25,000 IU (Webb et al., 2011); vastly exceeding the purported safe upper limit (SUL) of daily vitamin D_3 consumption. However, based on the findings from **Chapter 5** and **6,** a UVR exposure of 1.3 SED, to a white UK population wearing shorts and t-shirts provides the oral equivalent of 1,000 IU, well within the safe upper limit of daily vitamin D_3 consumption (Institute of Medicine, 2011).

7.5 Future directions

We chose to examine the influence of vitamin D on hepatitis B vaccine response following the second dose of the typical three dose series because there is widespread inter-individual variability in the magnitude of antibody response at this time (Szmuness et al., 1980). Further, the range of anti-HBs response following the second vaccine dose provides an experimental model to explore and identify factors which may influence the development of antibody response. In comparison, ~90% of individuals are considered to be sero-protected (anti-HBs ≥10 mIU/mL) following the third and final hepatitis B vaccine dose (Prather et al., 2012). However, we recognize that final antibody status following the full three-dose hepatitis B vaccine course was not captured in both **Chapter 4** and **Chapter 5**. Therefore, future studies may seek to confirm the importance of vitamin D status at the time of initial vaccination on sero-protective antibody status following the full hepatitis B vaccine course.

We successfully adopted and duplicated the methods of restoring and maintaining sufficient vitamin D concentrations, described in **Chapter 5**, to investigate how this might influence the development of new *in vivo* immune response. However, increasing vitamin D concentrations,

serum 25(OH)D, did not have any beneficial effect on secondary anti-HBs response. Given that we were only able to manipulate serum 25(OH)D following the initial hepatitis B vaccination, participants still displayed low vitamin D status at the time of the initial vaccine challenge. Therefore, we cannot rule out the possibility that vitamin D status at the time of initial vaccination may subsequently influence secondary anti-HBs response. Only one study has attempted to study how an intensive period (5 days) of UV treatment prior to vaccination may influence subsequent vaccine response (Sleijffers et al., 2001). In this instance despite observing a modulating influence of CHS response to the novel antigen Dipheylcyclopropenone (DPCP), no influence was observed on the antibody response following hepatitis B vaccination. However, it should be noted that the investigators were investigating potential immunosuppressive effects of more 'extreme' personal UV doses. In this instance, the authors included the use of fluorescent lamps with UV emission removed from that of sunlight, specifically exposure to personalised MED-related UV doses rather than a standardised challenge, and near total-body surface exposure, which is unlikely to occur in everyday life. Future studies should seek to raise vitamin D status prior to initial vaccination-using methods that replicate safe, practical government recommended summer sunlight, and daily oral vitamin D supplementation guidelines.

7.5.1 Effectiveness of high dose vitamin D supplementation

It is unclear whether larger vitamin D doses achieving greater than normal seasonal changes in serum 25(OH)D (e.g. > 100 nmol/L) would have beneficial effects on *in vivo* immunity. Although this appears as the next step in the sequence of studies, larger vitamin D doses may be ineffective because they will further increase 24, 25$(OH)_2$D. Evidence is now emerging that the 24, 25$(OH)_2$D metabolite may act at the VDR as a "blocking molecule" binding to the VDR and decreasing 1,25$(OH)_2$D activity (Curtis, Aenlle, Roos, & Howard, 2014). Since 24, 25$(OH)_2$D circulates in the blood at the concentrations 1000 times higher than 1,25$(OH)_2$D, the significantly higher prevailing 24, 25$(OH)_2$D concentrations are liable to contribute to significant decrease in the activity of the biologically active 1,25$(OH)_2$D. Given that 1,25$(OH)_2$D induced migration if DCs and the subsequent stimulation of antigen specific T and B cells has been shown to be important in the orchestration of antibody response to following diphtheria vaccination (Enioutina et al., 2008, 2009). It is possible that decreased 1, 25$(OH)_2$D as a consequence of sustained increases 24, 25$(OH)_2$D , might impair development of *in vivo*

antibody response following vaccination. It is important to note that the increase observed in $24,25(OH)_2D$ via the supplementation strategies employed in **Chapter 6** were far lower than previously shown in one recent publication; likely attributable to the employment of vitamin D doses in excess of the tolerable upper intakes (4,000 IU/day) (Owens et al., 2017). Of equal importance is the appreciation that higher doses of SSR and oral vitamin D_3 risk skin damage and vitamin D toxicity, respectively (Advisory Group on Non-ionising Radiation, 2017; Institute of Medicine, 2011).

Future research might also seek to prevent the decline in end of summer serum 25(OH)D, by commencing vitamin D supplementation in late summer or early autumn and continuing until spring (~6 months), rather than restoring vitamin D sufficiency from its winter nadir, as in **Chapter 6**. We recommend that 400 IU/day oral vitamin D_3 dose from the maintenance phase of **Chapters 5** and **6** would maintain end of summer vitamin D sufficiency in most. This approach corresponds with current IOM and EFSA recommendations (European Food Safety Authority, 2016; Institute of Medicine, 2011). Unlike simulated sunlight here is no time burden for an individual; no requirement for bulky irradiation cabinets; and oral vitamin D_3 supplementation is effective regardless of sun reactive skin type (Farrar et al., 2011).

7.5.2 Alternative measure of in vivo immunity

The measurement of antibody response following vaccination represents the culmination of a series of interactions of different immune cell types in various compartments occurring within a dynamic neuroendocrine milieu (Delves & Roitt, 2011). These antibodies are measured in serum, therefore yielding a quantifiable measure of the final product of this cascade of reactions, which has implications for both vaccine efficacy and as a marker of susceptibility to infections. However, at this point, it is unclear whether the magnitude of response to vaccination as utilised in **Chapters 4** and **6** of this thesis translates into altered risk of illness. Although illness, specifically URTI was monitored during training from the primary care medical records of participants. It is likely that true cases of URTI were underreported to medical personnel; making associative analysis between infection incidence and vaccination response not possible. It should also be appreciated that at present there is no ‘gold standard’ measure of *in vivo* immune competence (Albers et al., 2013). Therefore when selecting immunological assessment tools for research, it is advisable to give careful consideration to the

aspect(s) of immunity of interest, clinical relevance, biological significance and feasibility (Albers et al., 2013). For instance, investigations carried out by Cohen and colleagues have examined the effect of various social, behavioural, psychological and physiological stressors on susceptibility to illness; using the *in vivo* method of experimental infection with live pathogens, such as rhinovirus (Cohen et al., 2013; Cohen, Janicki-Deverts, Turner, & Doyle, 2015; Janicki-Deverts, Cohen, Turner, & Doyle, 2016; Prather, Janicki-Deverts, Hall, & Cohen, 2015). Although this method is highly clinically relevant, its use is somewhat restricted due to concerns associated with the risk to health of purposefully inducing symptoms of infection. An alternative approach as adopted in this thesis, is to use an *in vivo* vaccine challenge, such as hepatitis B. This method overcomes the risk associated with the above, but it is important to consider that the use of vaccine models as an *in vivo* measure of immunity also present a number of limitations. Most notably, adopting the vaccine model to study *in vivo* immune responses are somewhat restricted to clinical investigations conducted in medical settings, and ethical constraints may prevent application within laboratory settings without the medical expertise and facilities.

Recently, the utility of assessing experimental contact hypersensitivity (CHS) responses to the novel antigen Dipheylcyclopropenone (DPCP) has been demonstrated within an exercise immunology setting (Diment et al., 2015). Measures of *in vivo* immunity at the skin are particularly attractive because the skin constitutes the body's largest immunological organ, providing the first line of defence against pathogenic and environmental assaults (Clark, Ghosh, & Tonnesen, 2007). Experimental CHS, with contact sensitizers like DPCP, represent a primarily T cell-mediated immune response; coordinated by both the innate and adaptive immune systems. CHS is characterised by two distinct phases. Firstly, the sensitisation phase (induction) is initiated by application of known sensitising dose of DPCP. The magnitude of *in vivo* immune responsiveness is then quantified by measuring the responses elicited by secondary exposure to a dose series of the same antigen ~28 days after the initial sensitisation termed the elicitation phase (recall) (Christensen & Haase, 2012). The strength of the *in vivo* immune response is commonly quantified by the vigour of the resultant oedema (thickening) and erythema (redness), typically 48 h after applying the challenge. DPCP specifically is a specially synthesised chemical and is not encountered in the natural environment, therefore permitting the investigation of the influence of a variety of stressors i.e. micronutrient deficiencies on both the sensitisation and elicitation phase of the immune response. CHS with

the novel antigen DPCP is practical, safe and can be administered without the need for expensive equipment, invasive injections or blood sampling, making it a suitable immunological tool for both laboratory and field investigations. Moreover, the use of a novel antigen such as DPCP provides investigators with rigorous control over the timing and dose of sensitising exposure. Although it should be noted that this was not an issue in the current investigations, as our participants were naïve to the hepatitis B vaccine antigen and were all administered the same dose. Adapting the use of experimental allergens like DPCP into field settings or laboratory studies may allow the investigation of the influence of vitamin D on the orchestration of new *in vivo* immunity in settings where assessing antibody responses to vaccinations is not practical.

7.6 Perspectives

At present there is no consensus regarding classifications of vitamin D for optimal immune function. Despite this, current EFSA and IOM recommendations advise that avoiding vitamin D insufficiency is essential for bone and general health. Also a comprehensive review that summarizes findings for various studies attempting to evaluate threshold concentrations for serum 25(OH)D in relation to multiple health outcomes concluded that for all endpoints serum concentrations of 25(OH)D < 50 nmol/L are associated with adverse events. We add to this, by demonstrating that fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals (**Chapter 4**). This association was particularly strong in men, likely due to a higher overall incidence of vitamin D insufficiency (**Figure 4.2, 4.4**). In addition, both compromised anti-HBs response following vaccination coupled with fewer individuals achieving sufficient vitamin D status during the winter (15% of men and 26% of women; **Figure 4.2B**) provide further support for this threshold (**Chapter 4**). Further evidence of this is required to substantiate the importance of this threshold for optimal *in vivo* immune function. The findings presented in **Chapter 4** may also have implications that may extend to more populations who may be at greater risk of low vitamin D status; including the diseased and elderly. Indeed, it has been shown that vitamin D deficiency is highly prevalent in elderly CKD patients (Zitt et al., 2012). The same study also demonstrated that patients with vitamin D deficiency were more frequently non responders to the hepatitis B vaccination in comparison to patients who were not vitamin D deficient (Zitt et al., 2012).

As our vitamin D supplementation strategies were effective to eliminate vitamin D insufficiency and achieve vitamin D sufficiency in almost all, future studies may choose to employ these methods to definitively confirm or reject the possible benefits of vitamin D supplementation on *in vivo* immune outcomes, as discussed earlier. However, the data presented in **Chapter 5** and **6** of this thesis, could also be adapted to provide practical recommendations for young and healthy adults concerning how vitamin D sufficiency can be achieved. Mindful of key factors such as latitude and skin type, as little as 15 minutes of exposure to summer sunlight between 10am and 3pm wearing t-shirts and shorts on most days can achieve vitamin D sufficiency in most individuals. During winter months, when skin sunlight as a source of vitamin D is absent, dietary sources of vitamin D and vitamin D supplement become an important consideration. Evidence from **Chapter 5** & **6** demonstrates that consuming a 1,000 IU/day vitamin D_3 supplement during winter could restore vitamin D sufficiency in almost all participants. Consumption of this dose might be particularly relevant for those who are unlikely to achieve the safe summer sunlight guidance i.e. those who train indoors during the summer, or those required to wear clothing (for protective or religious reasons) that restricts skin sunlight exposure in the summer. Young adults who are able to meet the safe summer sunlight guidelines at the end of summer may benefit from consuming a 400 IU/day vitamin D_3 supplement through the autumn and winter to maintain vitamin D sufficiency.

7.6.1 Considerations for optimising vaccination response

The measurement of antibody response following vaccination has clear clinical implications for the protection against infections (vaccine efficacy) (Burns, 2012). However, given that antibody response was not assessed following the full vaccine series in this thesis; antibody response was used to assess the immune systems general ability to respond to an antigen. Although the majority of healthy individuals are observed to mount maximal antibody responses following the full 3 dose vaccine series (~90%) (Marsland et al., 2006). Evidence also suggests that responders to initial vaccine doses mount greater responses to subsequent doses (Prather et al., 2012). Therefore, it could be speculated that non-responders following initial doses may be at greater risk of being vaccine non-responders following the full dose series of the vaccine. A number of interventions have sought to improve the antibody response to vaccination e.g. administering vaccines in the morning, the addition of adjuvants to the

vaccine preparation and behavioural interventions i.e. aerobic exercise, with some success (Edwards et al., 2008; Edwards et al., 2006; Long et al., 2012). However, such interventions have been shown to have adverse effects and may be impractical in a public health setting (Gupta & Siber, 1995). Although adjusting the timing of vaccination i.e. restrict to summer when vitamin D status is typically higher, may be a simple, cost neutral and effective public health intervention to improve vaccine response. In practice this may not be practical, given that administering vaccines at specific times of the year may be undesirable in situations where maximal protection is required rapidly i.e. before military deployment, medical settings, holidays or competition in oversees countries where certain diseases are more prevalent. Instead, those who are more likely to have non-response i.e. male or those vaccinated during the winter whom are likely to have low vitamin D should be checked for seroprotective status (anti-HBs $\geq$ 10 mIU/mL following the full 3 course vaccine dose series) and subsequently offered additional booster vaccinations. The first step would identify those without immunisation and in turn improve overall immunisation rates across the population.

7.7 Main conclusions

The major conclusions from this thesis are;

1. 75% individuals who had baseline vitamin D status assessed during winter presented with vitamin D insufficiency (serum 25(OH)D < 50 nmol/L).
2. Fewer men and women who received their initial hepatitis B vaccination during the winter mounted a response to the hepatitis B vaccination (anti-HBs titres ≥10 mIU/mL) in comparison with men and women vaccinated during summer months.
3. The seasonal variation in secondary anti-HBs response mirrored the typically observed seasonal variation in serum 25(OH)D and $1,25(OH)_2D$ concentrations as well as the higher prevalence of vitamin D insufficiency during the winter.
4. Fewer secondary hepatitis B vaccine responders were observed among vitamin D insufficient individuals, and those with the lowest $1,25(OH)_2D$ concentrations. Of particular clinical interest is that vaccine responses in vitamin D insufficient men were lower than typically expected after two hepatitis B vaccine doses.
5. Safe and practical government recommended summer sunlight (SSR) and daily oral vitamin D_3 supplementation regimens resolved, within 5 weeks, the typical winter nadir in vitamin D concentrations; by achieving vitamin D sufficiency in ≥ 90% of participants. Specifically, exposure to SSR 3x weekly or supplementation with 1, 000 IU/day of vitamin D_3 was able to raise serum 25(OH)D concentrations by ~30 nmol/L and encouraged vitamin D sufficiency in ~95% of participants.
6. Furthermore, maintenance doses of 1 x weekly SSR exposures and 400 IU/day of vitamin D_3 administered over 8 weeks maintained elevated vitamin D status.
7. Despite successfully replicating the SSR and oral vitamin D supplementation regimens within a field setting, no subsequent influence was observed on the development of the secondary antibody response following hepatitis B vaccination.
8. In line with current IOM and EFSA recommendations for general health, young healthy adults should seek to maintain sufficient vitamin D status throughout the year by practicing safe summer sunlight during the summer, or daily oral vitamin D_3 supplementation with 400IU/day at the end of summer to prevent a winter decline in vitamin D status.

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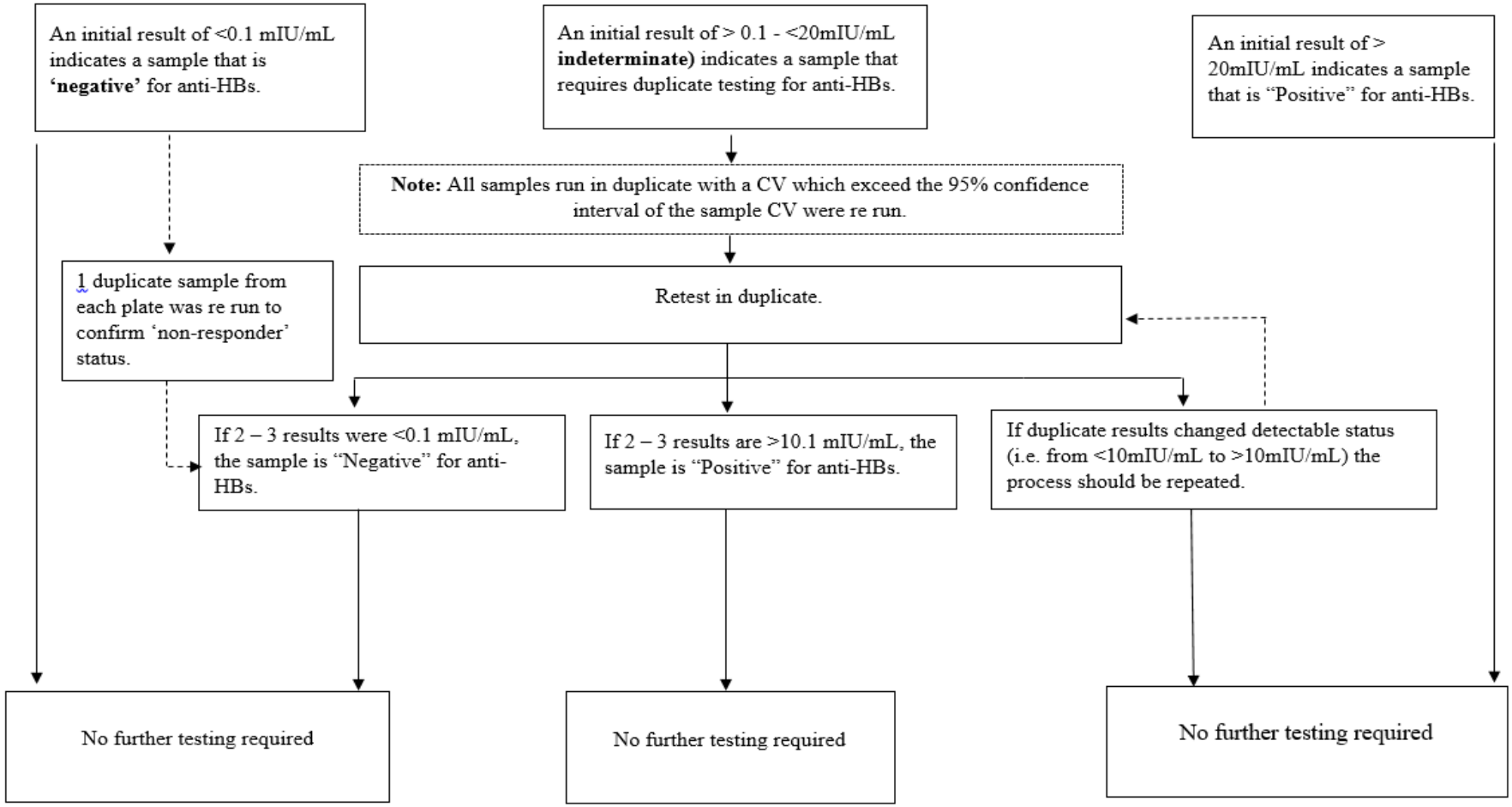
Appendix A: ELISA algorithm

Results for week 13 blood samples (B sample) were manually interpreted using the algorithm below.



Appendix B: DiaSorin anti-HBs enzyme immunoassay kit: Summary of assay results

Table 1. Summary of all assays run for study presented in Chapter 4. CV; coefficient of variation

ELISA plate	Date of assay	ELISA kit standards: CV	Quality control: Mean (9.0 – 17.7 mIU/mL)	Quality control: CV	Samples: CV
1	22-Feb-16	4.4	12.6	35.4	5.4
2	22-Feb-16	3.5	14.8	10.3	10.3
3	22-Feb-16	4.6	14.7	26.7	6.4
4	22-Feb-16	3.3	5.8	n/a	6.2
5	24-Feb-16	3.0	11.8	2.7	3.3
6	24-Feb-16	6.4	14.9	4.6	3.3
7	24-Feb-16	5.5	11.4	16.4	6.6
8	24-Feb-16	6.3	13.6	1.7	4.3
9	29-Feb-16	4.1	14.8	20.7	9.5
10	29-Feb-16	7.0	14.5	9.0	3.1
11	29-Feb-16	3.2	15.5	3.3	5.5
12	29-Feb-16	4.0	16.2	12.9	8.3
13	02-Mar-16	4.2	17.8	0.2	4.4
14	02-Mar-16	2.4	16.5	0.4	4.2

15	02-Mar-16	3.0	21.8	7.0	3.8
16	27-Jan-16	2.8	17.1	2.1	4.0
17	21-Apr-16	5.0	30.8	16.0	3.0
18	21-Apr-16	4.2	23.0	11.9	4.0
19	21-Apr-16	7.7	26.9	17.2	4.1
20	21-Apr-16	6.3	28.1	1.8	2.4
21	22-Apr-16	7.6	12.3	21.0	4.9
22	22-Apr-16	4.1	13.1	21.7	2.7
23	26-Apr-16	3.5	16.2	13.6	6.4
24	26-Apr-16	3.6	13.8	29.1	6.1
25	26-Apr-16	5.4	22.6	6.4	4.6
26	20-May-16	5.9	18.1	3.4	4.8

Table 2. Summary of all assays run for study presented in Chapter 6. CV; coefficient of variation

ELISA plate	Date of assay	ELISA kit standards: CV	Quality control: Mean (9.0 – 17.7 mIU/mL)	Quality control: CV	Samples: CV
1	10-Apr-17	6.7	24.9	9.8	2.6
2	11-Apr-17	2.8	27.9	11.9	8.7
3	11-Apr-17	5.1	31.0	3.6	3.6
4	18-May-17	5.7	18.4	11.9	1.0
5	18-May-17	5.3	18.7	6.2	2.9
6	18-May-17	3.8	18.0	1.7	4.3
7	18-May-17	2.5	22.0	11.4	3.3
8	19-May-17	3.7	22.7	17.3	5.2
9	19-May-17	3.8	19.2	1.5	3.6
10	19-May-17	3.0	24.9	13.3	5.1
11	19-May-17	14.2	21.4	17.2	3.3
12	19-May-17	2.2	17.6	13.5	9.5