

Predictors of vitamin D status in New Zealand adults and the effect of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> supplementation on 25-hydroxyvitamin D and parathyroid hormone concentrations

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## Abstract

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Vitamin D deficiency is associated with impaired calcium absorption leading to compensatory hyperparathyroidism, increased bone resorption and decreased bone integrity. The prevalence of low vitamin D status among New Zealanders is increasingly documented, and food fortification and/or supplementation strategies to meet physiological needs may be required. To this end, an examination of demographic, and lifestyle influences on vitamin D status are important for the development of population-specific health recommendations as is the determination of dose, frequency and form of vitamin D used for supplementation and/or food fortification.

There are two forms of vitamin D commonly used, ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>). Although public health recommendations do not discriminate between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, a number of studies using large, single oral dose preparations ranging from 50,000 to 300,000 IU have suggested that vitamin D<sub>2</sub> is less effective than vitamin D<sub>3</sub>. Lower dose clinical trials comparing daily administration of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> have yielded inconsistent results and have been limited by short study duration, small participant numbers, the effects of cutaneous production of vitamin D<sub>3</sub>, background dietary and supplemental vitamin D intake and body mass index. In an attempt to address some of these limitations, the goal of this thesis was to evaluate the effectiveness of a daily physiological dose of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> on serum 25(OH)D levels in healthy adults living in Dunedin, New Zealand (latitude 45°S). In addition, possible predictors of serum 25(OH)D status among this group of healthy adults were examined along with the relationship between serum 25 (OH)D and serum PTH concentrations.

To achieve the overall study goals, a placebo-controlled, double-blinded intervention trial was conducted in 95 participants, aged 18–50 y, who were randomly assigned to receive

either 1000 IU vitamin D<sub>3</sub>/d (n = 32), 1000 IU vitamin D<sub>2</sub>/d (n = 31) or placebo (n = 32) for 24 wk period beginning at the end of summer (March). Total serum 25(OH)D, as 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub>, and PTH were measured at baseline and at 4, 8, 12 and 24 wk. Baseline measurements of 25(OH)D and PTH concentrations (n = 95) were used to determine predictors of serum 25(OH)D status among participants, and to assess the relationship between serum 25(OH)D and PTH concentrations. Change over time in serum 25(OH)D and PTH concentrations were used to compare the potencies of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> supplementation over the winter.

At baseline, serum 25(OH)D concentrations ranged from 27.9 to 141.0 nmol/L with the geometric mean serum total 25(OH)D of 74.9 nmol/L. Seven (7.4%) participants had 25(OH)D concentrations below 50 nmol/L, six (6.3%) participants had a serum 25(OH)D concentration ranging between 30–50 nmol/L and one (1.1%) had a serum 25(OH)D < 30 nmol/L. None of the known predictors of vitamin D status such as age, body mass index (BMI), constitutive or facultative skin colour were significantly associated with serum 25(OH)D concentrations; however, mean serum 25(OH)D concentrations were 17 nmol/L higher in those participants taking a vitamin-D containing supplement in the 3 months prior to study commencement ( $P = 0.035$ ). In terms of the bone effects, serum PTH decreased as serum 25(OH)D concentrations increased with evidence of a plateau in PTH concentrations at a serum 25(OH)D of ~70 nmol/L.

Following 24 wk of supplementation, the vitamin D<sub>3</sub>-supplemented group demonstrated no significant change (t-test;  $P = 0.879$ ) in serum 25(OH)D concentration over the winter time course of the study, whereas mean (95% CI) 25(OH)D significantly decreased by 43 (35, 50) nmol/L ( $P < 0.001$ ) in the placebo group and by 22 (14, 30) nmol/L in the vitamin D<sub>2</sub>-supplemented group ( $P < 0.001$ ). There was no evidence of a statistically significant change in serum PTH concentrations observed between the groups ( $P = 0.646$ ),

nor was there a change in 25(OH)D<sub>3</sub> throughout the study in Vitamin D<sub>3</sub> treated participants. As expected, participants assigned to both the vitamin D<sub>2</sub> and placebo groups experienced a significant decline in 25(OH)D<sub>3</sub> concentration over the winter months relative to the vitamin D<sub>3</sub> supplemented group; however, baseline adjusted serum 25(OH)D<sub>3</sub> was significantly lower [7.1 (0.8, 13.3) nmol/L] in the vitamin D<sub>2</sub> group compared to the placebo group ( $P = 0.028$ ).

In conclusion, vitamin D<sub>3</sub> is more effective than vitamin D<sub>2</sub> in maintaining summertime 25(OH)D concentration over the winter period. This difference may be partly explained by the negative effect of vitamin D<sub>2</sub> ingestion on serum 25(OH)D<sub>3</sub> levels. The assumption of vitamin D<sub>2</sub> and D<sub>3</sub> equivalence from not only a physiological but also a therapeutic basis should be reconsidered, particularly in light that the dose employed in our study is commonly used in over-the-counter dietary supplements. Furthermore, the use of vitamin D-containing supplements significantly impacts vitamin D status, even when UV synthesis is high.

## Preface

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This randomised, controlled double-blinded trial was conducted in the Department of Human Nutrition at the University of Otago, Dunedin, New Zealand. Dr. Lisa Houghton, the candidate's supervisor, was responsible for the conception and overall design of the study, securing the required funding, and obtaining ethical approval. Michelle Harper performed the laboratory analysis of serum 25-hydroxyvitamin D. The candidate, Victoria Logan (VL) conducted the research along with research assistant, Meredith Rose (Department of Human Nutrition, University of Otago).

### ***Chapter 3: Predictors of vitamin D status and its association with parathyroid hormone in healthy New Zealand adults***

Biochemical baseline measurements were used to identify possible predictors of serum 25-hydroxyvitamin D status in healthy adults and to describe the relation of 25-hydroxyvitamin D with parathyroid hormone levels.

Specifically, the candidate (VL) was responsible for:

- Entering diet records to determine average daily calcium intake.
- Laboratory analysis of parathyroid hormone concentrations using an automated electrochemiluminescence immunoassay.
- Data entry, data cleaning and carrying out the statistical analysis and interpretation of results in consultation with Dr Lisa Houghton and Mr Andrew Gray, biostatistician (Department of Preventive and Social Medicine, University of Otago).
- Presentation of the results, discussion and preparation of thesis.

**Chapter 4: *Vitamin D<sub>3</sub> is more effective than vitamin D<sub>2</sub> in maintaining  
vitamin D status over the winter months***

The randomised controlled trial assessed the effect of two forms of vitamin D supplementation over the winter period in Dunedin, New Zealand.

Specifically, the candidate (VL) was responsible for:

- Organising participants and conducting 3 of the 5 clinic visits, including the collection of sociodemographic data, anthropometric measurements, skin colour assessment and the processing and storage of blood samples.
- Obtaining an amendment to ethical approval to obtain an additional blood sample for the identification of vitamin D binding protein genetic polymorphisms and assessment of mood (results not reported).
- Determining serum parathyroid hormone concentration using an automated electrochemiluminescence immunoassay (all five clinic visits; approximately 450 samples).
- Data entry, data cleaning and carrying out the statistical analysis and interpretation of results in consultation with Dr Lisa Houghton and Mr Andrew Gray, biostatistician (Department of Preventive and Social Medicine, University of Otago).
- Presentation of the results, discussion and preparation of thesis.
- Drafting of manuscript entitled “Vitamin D<sub>3</sub> is more effective than vitamin D<sub>2</sub> in maintaining serum 25-hydroxyvitamin D status over the winter months” submitted to the Journal of Clinical Endocrinology and Metabolism.

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## List of Abbreviations

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1,25(OH) <sub>2</sub> D	1 $\alpha$ ,25-dihydroxyvitamin D
24,25(OH) <sub>2</sub> D	24,25-dihydroxyvitamin D
25(OH)D	25-hydroxyvitamin D
5-HT	Serotonin
AI	Adequate intake
AUC	Area under the curve
BMD	Bone mineral density
BMI	Body mass index
CI	Confidence interval
CNS	Central nervous system
CPB	Competitive protein binding
CYP	Cytochrome P450
DBP	Vitamin D-binding protein
DRI	Dietary Reference Intake
EAR	Estimated Average Requirement
HPLC	High-performance liquid chromatography
ITA	Individual Typology Angle
IU	International unit
LASA	Longitudinal Aging Study Amsterdam
LC-MS	Liquid chromatography-mass spectrometry
LOWESS	Locally weighted scatterplot smoother line
MS	Multiple sclerosis
NHANES	National Health and Nutrition Examination Survey
OR	Odds ratio
PO <sub>4</sub> <sup>-</sup>	Phosphate
PTH	Parathyroid hormone
RCT	Randomised controlled trial
RDA	Recommended Dietary Allowance
RIA	Radioimmunoassay
RNI	Recommended nutrient intakes
RR	Relative risk
SAD	Seasonal affective disorder
SNP	Single nucleotide polymorphism
T1DM	Type 1 diabetes mellitus
VDR	Vitamin D receptor
VDR <sub>mem</sub>	Vitamin D membrane receptor
VDR <sub>nuc</sub>	Vitamin D nuclear receptor
UK	United Kingdom
UL	Upper level of intake
USA	United States of America
UVB	Ultra-violet B
Wk	Week
WMD	Weighted mean difference

# 1 Introduction

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Vitamin D (calciferol), through its biologically active form, 1,25-dihydroxyvitamin D, plays a key role in the intestinal absorption of calcium and is essential for maintaining calcium:phosphate homeostasis and bone metabolism (Wasserman, 2005). Although less well elucidated, recent evidence has revealed a diverse range of biological actions of vitamin D beyond that of bone health, which suggest a connection between vitamin D and several chronic diseases, including various cancers, cardiovascular disease, diabetes and multiple sclerosis (Adams & Hewison, 2010; Holick, 2007; Munger et al., 2006; Scientific Advisory Committee on Nutrition, 2007). The prevalence of suboptimal vitamin D status is difficult to assess as controversy currently exists with regard to the interpretive criteria for 25-hydroxyvitamin D [25(OH)D] level across the lifecycle and among subgroups (Hollis, 2005). Limitations in assays and lack of methodological standards have also made it difficult to reach a consensus pertaining to vitamin D status cut-offs. The presence of signs/symptoms that characterise skeletal disease states (i.e. rickets and osteomalacia) have been used to define vitamin D deficiency, typically occurring at circulating 25(OH)D concentrations of less than 30 nmol/L (12 ng/ml) (Institute of Medicine, 2010). Additional information on the functional role of vitamin D in bone health may be obtained by evaluating the 25(OH)D concentration at which serum parathyroid hormone (PTH) concentration is maximally suppressed. Several researchers have suggested 25(OH)D thresholds > 50 nmol/L (20 ng/ml) as optimal, indicative of adequate vitamin D status, with levels > 75 nmol/L not consistently associated with increased benefit (Institute of Medicine, 2010). In New Zealand the prevalence of serum 25(OH)D < 50 nmol/L is around 50% (Rockell et al., 2005; Rockell et al., 2006).

The main source of vitamin D is cutaneous synthesis resulting from exposure to sunlight (ultraviolet B rays, UVB) (Holick, 2004a). Few foods naturally contain significant amounts of vitamin D and the food supply in many countries, including New Zealand, remains largely unfortified. The cutaneous generation of vitamin D is altered by multiple factors including latitude, season, skin pigmentation, age, obesity and personal factors relating to exposure to sunlight, such as time spent outdoors, clothing coverage and sunscreen use (Webb, 2006). At latitudes above 37°N and below 37°S, there is little UVB radiation during winter, thus seasonal declines in vitamin D status are observed (Chen, 1999; Norman, 1998; Webb et al., 1988). During winter in New Zealand (41°S), UVB radiation is not strong enough to facilitate the generation of sufficient vitamin D and the already high risk of melanoma skin cancer makes it inappropriate to suggest that individuals increase their exposure to sunlight or use less sunscreen. Consequently, the only way to improve vitamin D status safely and effectively is by increased fortification of food products with vitamin D and/or the administration of dietary supplements

There are two main forms of vitamin D, vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol) which are officially considered to be equivalent, however the efficacy of these two forms in improving vitamin D status, by raising 25(OH)D, has been queried. The suggestion of possible differences with regard to their equivalence was first raised in 1930, when cod liver oil (containing vitamin D<sub>3</sub>) was found to be more effective than Viosterol (vitamin D<sub>2</sub> containing oil) at treating rickets (Hess et al., 1930). However, subsequent studies undertaken in the 1940s produced unclear results, and early biomarker studies using 25(OH)D as a biomarker indicated that vitamin D<sub>3</sub> was more effective, but were also inconclusive (Hartwell et al., 1987; Park, 1940; Tjellesen et al., 1986; Whyte et al., 1979).

More recently, some studies suggest that both forms are bioequivalent (Holick et al., 2008; Rapuri et al., 2004), while others suggest that vitamin D<sub>3</sub> is the more potent form (Armas et al., 2004; Harwood et al., 2004; Leventis & Kiely, 2009; Trang et al., 1998). The interpretation of these studies has been weakened by the confounding effects of seasonal changes in 25(OH)D concentration, short study duration, differences that arise from type (bolus or daily) and route (oral or intramuscular) of administration, small participant numbers, differing vitamin D status at baseline and not controlling for body mass index (BMI). Large intervention studies that control for confounding factors, have sufficient sample sizes to detect statistical differences and are of adequate duration are required to confirm whether there are differences in potency between the two forms of vitamin D, particularly in the area of daily supplement regimens.

Hence, the overall goal of this study was to compare the efficacy of the two forms of vitamin D in maintaining serum 25(OH)D status over the winter period in Dunedin, New Zealand (46°S). We also aimed to identify predictors of serum 25(OH)D status in our group of healthy adults residing in Dunedin, New Zealand and to assess the relationship between serum 25(OH)D and PTH. This was achieved using data from a placebo-controlled, double-blinded supplementation trial conducted in 95 healthy adult participants, who were randomly assigned to receive either 1000 IU vitamin D<sub>3</sub>/d (n = 32), 1000 IU vitamin D<sub>2</sub>/d (n = 31) or placebo (n = 32) for 24 wk period beginning at the end of summer (March). The specific objectives of the research were to:

1. Determine serum 25(OH)D status and to identify predictors of serum 25(OH)D status among participants using baseline data
2. Examine the relationship between serum PTH and serum 25(OH)D at baseline for evidence of a threshold or plateau .

3. Evaluate the effect of a daily physiological dose of vitamin D<sub>2</sub> or vitamin D<sub>3</sub> on serum 25(OH)D status and PTH concentrations over 24 wk intervention period.

## 2 Literature Review

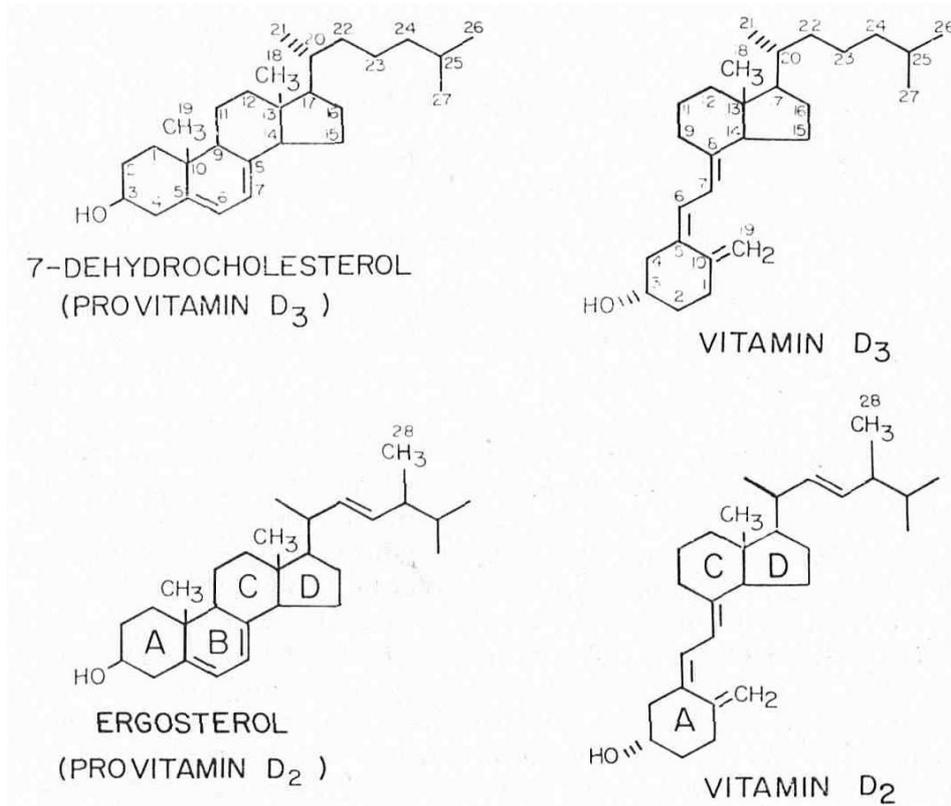
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### 2.1 Vitamin D

#### 2.1.1 Chemical structure and properties

Vitamin D is a generic term, which collectively refers to a molecule exhibiting a ring structure derived from the cyclopentanoperhydrophenathrene ring structure for steroids (**Figure 2.1**) (Norman, 2008). Vitamin D precursor (provitamin D), a rigid, 4-ringed structure with two conjugated double bonds at carbons 5 and 7 (Holick, 2004a), is converted to previtamin D, resulting in an open seco-steroid, with a broken carbon-carbon bond in ring B between carbons 9 and 10 (Holick, 1981; Norman, 2008). Analogues of vitamin D have different precursors and side-chains (Norman, 2008), enabling them to interact with a variety of proteins (Okamura et al., 1992), which accounts for the myriad of biological actions associated with vitamin D.

The major physiologically relevant forms of vitamin D are vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol). Vitamin D<sub>2</sub> is of plant origin, and is produced by the action of ultraviolet light on the plant steroid ergosterol. Vitamin D<sub>3</sub> is synthesised in the skin of humans and animals produced through the action of ultraviolet B (UVB) light on 7-dehydrocholesterol (Holick, 1981; Holick, 2004a; Norman, 2008). Structurally, the only difference between vitamin D<sub>2</sub> and vitamin D<sub>3</sub> is in their side chains, in which vitamin D<sub>2</sub> contains both an additional double bond between carbons 22 and 23 and a carbon 24-methyl group (Holick, 1981).



**Figure 2.1:** Chemical structures of vitamins D<sub>2</sub> and D<sub>3</sub>, and their precursors (Holick, 1981)

### 2.1.2 Cutaneous synthesis

Sunlight is the primary source of vitamin D, with 90–95% of most humans' circulating vitamin D coming from sunlight exposure (Holick, 2003a). When the skin is exposed to sunlight in the UVB range (290-315 nm) (MacLaughlin et al., 1982), 7-dehydrocholesterol, which is present in the epidermis is converted to previtamin D<sub>3</sub> (precholecalciferol). Previtamin D<sub>3</sub> then undergoes thermal isomerization (involving a hydrogen shift) to form vitamin D<sub>3</sub> (Dusso et al., 2005; Holick, 1981; Okamura et al., 1992). Following this conversion, vitamin D<sub>3</sub> selectively exits the plasma membrane of skin cells (Holick, 1981; Holick, 1996) and is transported to the liver bound to vitamin D binding protein (DBP) (Holick, 2008). Vitamin D binding protein has virtually no affinity for previtamin D<sub>3</sub>, and

thus previtamin D<sub>3</sub> remains in the epidermis to continue its thermal isomerization (Holick, 1981; Holick, 2004a).

Sunlight regulates the production of vitamin D<sub>3</sub> through its ability to photodegrade both previtamin D<sub>3</sub> and vitamin D<sub>3</sub>. Following prolonged sun exposure, production of vitamin D<sub>3</sub> reaches a plateau at a concentration of 10–15% of the original 7-dehydrocholesterol concentration. Once previtamin D<sub>3</sub> production is maximised, further UVB exposure results in the conversion of previtamin D<sub>3</sub> to biologically inactive photoisomers: lumisterol and tachysterol. The DBP has little binding affinity for either tachysterol or lumisterol, so these products also remain in the skin rather than entering the circulation (Holick, 1981; Holick et al., 1981). Upon depletion of previtamin D<sub>3</sub> stores, exposure of lumisterol and tachysterol to UV radiation can thermally isomerise either of these products back to previtamin D<sub>3</sub>. Furthermore, if vitamin D<sub>3</sub> is not transported into the circulation, it is photisomerised to suprastol I, suprastol II or 5,6-*trans*-vitamin D<sub>3</sub> (Holick, 1994). Hence, prolonged sun exposure never results in excessive or toxic production of vitamin D<sub>3</sub>, as the concentration of vitamin D precursors reach equilibrium.

### **2.1.3 Dietary sources and absorption**

There are relatively few foods that are natural sources of vitamin D (Holick, 2004a) (**Table 2.1**). Most foods are primarily of animal origin, with oily fish (salmon, sardines, and mackerel) and egg yolk being among the most commonly consumed sources. For example, salmon contains 400–1280 IU/100 g (Lu et al., 2007; Nakamura et al., 2002), making it a particularly good source of vitamin D. The content of vitamin D in egg yolk is variable, ranging from 10 to 50 IU/yolk depending on the vitamin D content of the feed (Holick, 2004a; Ovesen et al., 2003). Wild and irradiated mushrooms are the only non-animal source

of vitamin D. They contain ergosterol, which is converted to vitamin D<sub>2</sub> following UV exposure. The vitamin D content of mushrooms differs extensively, depending on irradiation dose and mushroom species, with values ranging from 8–1200 IU/100 g fresh mushrooms (Mattila et al., 2002).

**Table 2.1: Vitamin D content of selected food sources**

Food	Food content (IU/100 g) <sup>a</sup>	Serving size	Amount vitamin D <sub>3</sub> per serve (IU/ µg)
Cod liver oil	9 080	1 tablespoon (15ml)	1360/ 34
Salmon (canned, pink)	468	100g	466/ 11.7
Tuna, canned in oil	236	100g	236/ 5.9
Egg, raw, whole	36	46g	16/ 0.4
Beef liver, raw	16	100g	16/ 0.4
Chicken breast, cooked	44	100g	44/ 1.1

<sup>a</sup> Data from USDA nutrient database (United States Department of Agriculture, 2009) with exception of chicken breast, cooked obtained from Hill et al. (Hill et al., 2004)

Dairy products, edible oil table spreads or margarine, juices and breakfast cereals are commonly fortified with vitamin D (Prentice, 2008). Permissible levels of fortification vary widely from country to country, and fortification practices are often dependent on the food industry rather than government policy. In Canada and Finland, there is mandatory fortification of milk and margarine (Calvo et al., 2004; Scientific Advisory Committee on Nutrition, 2007) and of margarine only in Australia (Food Standards Australia New Zealand, 2009) and the United Kingdom (Scientific Advisory Committee on Nutrition, 2007). The United States currently has no mandatory fortification although vitamin D is considered to be a safe ingredient and may be voluntarily added to milk, juice, infant formula and breakfast cereals at controlled levels (Calvo et al., 2004). In New Zealand, dairy products (yoghurts, reduced fat and skim milks), margarine and fortified foods (cereal-based

beverages, fruit/vegetable juice) can also be voluntarily fortified with vitamin D (Food Standards Australia New Zealand, 2009). Vitamin D intakes are higher in countries where there is widespread and/or mandatory fortification of foods with vitamin D, compared to countries where there is little vitamin D fortification (Calvo et al., 2005). Nowson and Margerison (2002) found that dietary intakes are 80–120 IU per day (2–3 µg/d) higher in countries where both milk and margarine are fortified with vitamin D than in countries where only one food is fortified.

Dietary vitamin D supplements are available as either synthesised vitamin D<sub>2</sub> or D<sub>3</sub>, or as concentrated fish liver oils. Cod liver oil contains substantial amounts of vitamin D, at approximately 100 IU/ml (Blackmores Australia, 2009; Healtheries, 2009; Linday et al., 2004) and was provided to children for its anti-rachitic effects years before vitamin D was chemically identified (Holick, 2004a). In New Zealand, most supplemental vitamin D is vitamin D<sub>3</sub>, with high dose over-the-counter supplements providing 1000 IU/capsule (25 µg) (Blackmores Australia, 2009; Thompson's, 2009). Multivitamin and calcium supplements may also contain vitamin D, albeit in smaller amounts.

Digestion of dietary and supplemental vitamin D is generally aided by emulsification and the presence of bile salts. Absorption occurs mainly in the jejunum via nonsaturable passive diffusion (Basu & Donaldson, 2003; Hollander, 1976). Once inside the enterocyte vitamin D is incorporated into chylomicrons and transported into the lymphatic system (Hollander, 1981). Both dietary and endogenously produced vitamin D then enters the venous circulation via the thoracic duct and is transported to the liver, bound to DBP. Absorption of vitamin D is not dependant on status and is normally high across all stages of

life (Clemens et al., 1986); however, it is decreased when dietary intake of fat is reduced (Ovesen et al., 2003).

It has been demonstrated that vitamin D<sub>3</sub> is absorbed into the lymph at a faster rate than D<sub>2</sub>, and is also transported more rapidly (Schachter et al., 1964). Hydroxylated dietary vitamin D [25-hydroxyvitamin D, 25(OH)D] also appears to be more bioavailable than unhydroxylated vitamin D (Sitrin & Bengoa, 1987) due to its more polar nature, which enables faster and more efficient absorption, as it is not as dependent on bile acids for absorption. This more polar nature also facilitates the absorption of 25(OH)D via the portal circulation with direct transport to the liver (Davies et al., 1983; Maislos et al., 1981).

#### **2.1.4 Metabolism**

Whether dietary or endogenously produced, vitamin D must undergo further metabolism before it becomes biologically active (Holick, 1996). The first step in the metabolic activation of vitamin D occurs in the liver with hydroxylation at the carbon 25 position, resulting in the production of 25(OH)D (calcidiol) (Holick, 1981). 25-hydroxyvitamin D is then released into circulation mostly bound to DBP (Norman, 1998). There are several hepatic cytochrome P450 (CYP) enzymes that can hydroxylate vitamin D, including the extensively studied CYP27A1 (vitamin D-hydroxylase) (Bikle, 2009). However, evidence from CYP27A1 'knockout' mice demonstrates minimal change in 25(OH)D levels (Rosen et al., 1998), which suggests this is not the main enzyme responsible (Dusso et al., 2005). Alternatively, CYP2R1 may be the primary mediator for this hydroxylation, as it is found mainly in the liver, is capable of hydroxylating both D<sub>2</sub> and D<sub>3</sub> (Cheng et al., 2003) with similar efficiency (Strushkevich et al., 2008), and mutations of this gene have been observed in patients with rickets (Cheng et al., 2004). This first

hydroxylation step exhibits first order kinetics, whereby 25(OH)D levels increase in proportion to the concentration of vitamin D (Holick, 1981). Plasma 25(OH)D levels are therefore a good reflection of cumulative effects of sunlight exposure and dietary intake (Bikle, 2009; Holick, 2008).

The second hydroxylation of vitamin D occurs primarily in the kidneys and forms the biologically active 1 $\alpha$ ,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D or calcitriol] via 25(OH)D-1 $\alpha$ -hydroxylase enzyme CYP27B1 (Fraser & Kodicek, 1970); or 24,25-dihydroxyvitamin D (24,25(OH)<sub>2</sub>D) via 24-hydroxylase enzyme CYP24 (Prentice et al., 2008). The kidney then secretes 1,25(OH)<sub>2</sub>D into circulation whereby it is exported bound to DBP, and transported to tissues. The half-life of 1,25(OH)<sub>2</sub>D in circulation is around 4–6 hours, compared to the relatively longer half-life of 25(OH)D of at least two weeks (Holick, 2004a; Lund et al., 1980; Wu et al., 2003).

The conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D was first believed to occur only in the kidney. However, more recent studies have shown that other tissues in the body, such as the prostate and colon are able to express 25(OH)D-1 $\alpha$ -hydroxylase and thus are also capable of producing 1,25(OH)<sub>2</sub>D (Cross et al., 2001; Schwartz et al., 1998; Tangpricha et al., 2001). 1,25-dihydroxyvitamin D produced in these tissues has cell-specific functions regulated by the autocrine/paracrine system (Hewison et al., 2004). Tissue-specifically produced 1,25(OH)<sub>2</sub>D is generally not secreted into the circulation.

Unlike 25(OH)D, circulating levels of 1,25(OH)<sub>2</sub>D are tightly regulated to maintain calcium homeostasis (Norman, 2008). Four regulatory factors are involved: serum concentrations of calcium and phosphate (PO<sub>4</sub><sup>-</sup>), 1,25(OH)<sub>2</sub>D itself, parathyroid hormone

(PTH) and foetal growth factor-23. Production of  $1,25(\text{OH})_2\text{D}$  by the kidneys increases under conditions of low circulating  $1,25(\text{OH})_2\text{D}$  concentrations, and is reduced under high concentrations (Norman, 2008). Parathyroid hormone is released in response to the body's demand for calcium and phosphorous, and stimulates the renal production of  $1,25(\text{OH})_2\text{D}$ .

## 2.2 Biological Function of Vitamin D

### 2.2.1 Vitamin D receptors

The cellular and physiological actions of  $1,25(\text{OH})_2\text{D}$  are mediated via interaction with vitamin D receptors (VDR). These receptors are located in more than 30 different tissues including the intestine, bone (osteoclasts), heart, breast, prostate, pancreas and kidney (Bouillon et al., 1995; Holick, 2004b). The presence of VDR in tissues that are not involved in bone mineralisation and calcium homeostasis suggests that these analogues have a role in cancer prevention, controlling various endocrine systems and the haemopoietic system, and may have immunomodulatory effects (Dusso et al., 2005; Okamura et al., 1995).

$1\alpha,25$ -dihydroxyvitamin D, like other steroid hormones, influences these responses through its interaction with vitamin D nuclear receptors ( $\text{VDR}_{\text{nuc}}$ ) and with membrane receptors ( $\text{VDR}_{\text{mem}}$ ) (Brown et al., 1999; Norman, 2005; Norman et al., 2004). The two receptors preferentially interact with each of the two stereoisomers of  $1,25(\text{OH})_2\text{D}$ . Ligand shaped *6-s-cis*  $1,25(\text{OH})_2\text{D}$  is optimal for rapid membrane responses ( $\text{VDR}_{\text{mem}}$ ), whereas  $\text{VDR}_{\text{nuc}}$  (genomic responses) favour the bowl shape of *6-s-trans*  $1,25(\text{OH})_2\text{D}$  (Norman, 2005). Tissues that express the VDR are therefore able to produce biological responses depending on the availability of sufficient amounts of vitamin D (Norman, 2008), accounting for the varied role vitamin D plays in health. The VDR protein contains 427 amino acids, with six areas – variable regions (A and B domains), a DNA-binding domain (C-domain), the hinge region (D-domain), a ligand-binding domain (E-domain) and a transcriptional activating-domain (F-domain) (DeLuca, 2004).

Free  $1,25(\text{OH})_2\text{D}$  can enter the cells by passive diffusion, or in some cell types, such as liver and kidney,  $1,25(\text{OH})_2\text{D}$  is taken up by facilitated diffusion. It is then available to interact with a specific high-affinity nuclear receptor protein ( $\text{VDR}_{\text{nuc}}$ ) (Norman, 2005). The unoccupied receptor interacts with its associated ligand  $1,25(\text{OH})_2\text{D}$ , to form a ligand-receptor complex, which induces conformational changes in the receptor protein (Norman, 2008). These changes enable the complex to interact with transcriptional proteins, facilitating the role of vitamin D in gene transcription. The complex acts through vitamin D-responsive elements, found near the start of the target gene (promoter region), in conjunction with coactivators, corepressors and other proteins (Bikle, 2009; Carlberg, 2003; DeLuca, 2004). It is believed that  $1,25(\text{OH})_2\text{D}$  directly affects about 200 genes (out of the ~20 500 genes in the human genome) (Carlberg, 2003), reflecting the widespread distribution of VDR and  $25(\text{OH})\text{D}-1\alpha$ -hydroxylase throughout various tissues in the human body.

The responses that arise via membrane initiated pathways ( $\text{VDR}_{\text{mem}}$ ) are viewed as ‘rapid responses’ because they occur too fast to simply be a result of  $1,25(\text{OH})_2\text{D}$  binding to  $\text{VDR}_{\text{nuc}}$  (Norman, 2005).  $1\alpha,25$ -dihydroxyvitamin D interacts with caveolae (membrane invaginations)-associated VDR, which activate second messenger systems (Norman, 2008). This binding of vitamin D to VDR triggers a change in the concentration of the receptive second messenger, which then mediates changes in the cell through a series of reactions (Campbell & Farrell, 2003). Important cellular messenger responses that  $1,25(\text{OH})_2\text{D}$  is involved in include the activation of protein kinase C and phosphatidylinositol 3-kinase, which are involved in controlling mineral formation in chondrocytes and vascular smooth muscle growth and calcification, respectively (Boyan et al., 1998; Rebsamen et al., 2002; Schwartz et al., 2002). Through the mitogen-activated protein-kinase second messenger

system, 1,25(OH)<sub>2</sub>D is involved the phosphorylation of transcription factors that are essential for cell differentiation (Campbell & Farrell, 2003; Song et al., 1998).

Different allelic variants (polymorphisms) of the VDR gene occur naturally in the human population, with different races and ethnicities having distinct variants (Uitterlinden et al., 2004). Expression of different polymorphisms may be associated with decreased bone density (Gennari et al., 2002; Huang & Kung, 2006; Zmuda et al., 2000), hyperparathyroidism (Krebs & Arnold, 2002; Zmuda et al., 2000) and susceptibility to cancers (Davis, 2008; Rukin & Strange, 2007; Slattery, 2007).

### **2.2.2 Mineral homeostasis**

One of the foremost functions of vitamin D is the maintenance of serum calcium and phosphate in the normal physiological range, ensuring mineralisation of bone and prevention of hypercalcaemic tetany (Holick, 1996). The calciotropic hormones 1,25(OH)<sub>2</sub>D, PTH and calcitonin tightly regulate serum calcium through both direct and indirect actions on the intestine, kidney and bone (Wasserman, 2005).

Vitamin D is required for the optimisation and regulation of intestinal calcium absorption. Intestinal calcium absorption involves two distinct mechanisms: a saturable transcellular movement that follows Michaelis-Menten-type kinetics and a non-saturable paracellular movement, which is directly dependent on the luminal concentration of calcium (Wasserman, 2005). Under conditions of low calcium intake, it is generally accepted that the active transcellular route is stimulated, whereas during periods of high calcium supply the bulk of calcium is absorbed via the paracellular pathway (Bronner, 2009). There is

increasing evidence that vitamin D enhances the paracellular absorption of calcium; while  $1,25(\text{OH})_2\text{D}$  is primarily involved in the former transcellular pathway (Wasserman, 2005).

Transcellular intestinal calcium transport is a multi-step process consisting of at least three phases (Bouillon et al., 2003; Bronner, 2009; Wasserman, 2005): (1) calcium entry across the brush border; (2) intracellular calcium transport and (3) calcium extrusion into the blood stream at the basolateral membrane of epithelial cells. It is well established that  $1,25(\text{OH})_2\text{D}$  is essential in the first two steps of this transport. In the first step, calcium entry requires the presence of channels. Vitamin D plays a role in the expression of calcium entry channels through the interaction of VDR with  $1,25(\text{OH})_2\text{D}$  (Holick, 2007; Wood et al., 2001).  $1,25$ -dihydroxyvitamin D is also involved in the induction of cytosolic transporting protein, calbindin $\text{D}_{9k}$  which aids the transport of calcium through the interior of the cell (Christakos et al., 1992; Slepchenko & Bronner, 2001). Of all presently known genes involved in intestinal calcium absorption, those coding for the epithelium calcium channels are the most responsive to  $1,25(\text{OH})_2\text{D}$  concentrations (Bouillon et al., 2003).

When dietary calcium is insufficient, a decrease in serum calcium concentration occurs and induces a calcium-sensitive protein in the parathyroid glands to enhance synthesis and secretion of PTH. Parathyroid hormone increases  $1,25(\text{OH})_2\text{D}$  production through the stimulation of the CYP27B1 gene that produces the  $1\alpha$ -hydroxylase, leading to synthesis of  $1,25(\text{OH})_2\text{D}$ , which then activates the three sites of calcium mobilisation (bone, kidney and intestine) (DeLuca, 2008).  $1,25$ -dihydroxyvitamin D signals intestinal absorption of calcium, and acts in conjunction with PTH to increase both renal distal tubular reabsorption of calcium (Holick, 1996; Yamamoto et al., 1984) and the mobilisation of calcium from the bone (DeLuca, 2004). The mobilisation of calcium occurs following the action of

1,25(OH)<sub>2</sub>D on osteoblasts, producing receptor activator nuclear factor- $\kappa$ B ligand (Suda et al., 2003), which then stimulates osteoclastogenesis and activates resting osteoclasts for bone resorption (Holick, 1996; Suda et al., 2003).

Phosphorous is also an essential component of cellular metabolism and skeletal mineralisation (as calcium phosphate). Ingested phosphorous is absorbed by the small intestine and deposited in the bone. Renal reabsorption of phosphorous occurs according to need (Tenenhouse & Portale, 2005). For example, a low phosphorous diet (leading to hypophosphataemia) associated with a reciprocal increase in circulating serum calcium, which suppresses PTH and decreases renal excretion of PO<sub>4</sub><sup>-</sup> while increasing calcium excretion. Hypophosphataemia increases proximal tubular reabsorption of PO<sub>4</sub><sup>-</sup> and stimulates renal synthesis of 1 $\alpha$ -hydroxylase, causing an increase in 1,25(OH)<sub>2</sub>D, which enhances intestinal absorption of both phosphorous and calcium, and the mobilisation of each from bone. This homeostatic process normalises cellular phosphate concentration, with a minimal effect on serum calcium concentration (Tenenhouse & Portale, 2005). Hyperphosphataemia produces the opposite effects on bone, kidney and intestine, decreasing plasma phosphate concentrations towards normal values.

### **2.2.3 Implications of low vitamin D status on skeletal health**

The role of vitamin D in relation to skeletal health has been extensively studied. Coordinated actions of calcium and 1,25(OH)<sub>2</sub>D are essential for growth plate development, and 1,25(OH)<sub>2</sub>D and VDR are needed for optimal osteoblastic bone formation and osteoclastic bone resorption (Dusso et al., 2005). The most well known consequences of vitamin D deficiency are rickets in children and osteomalacia in adults.

## **Rickets**

Rickets is a consequence of impaired bone mineralisation in children that occurs primarily due to inadequate calcium or phosphorous at the epiphyseal plates. Calcium may be limited either by inadequate dietary intake or inadequate absorption; while phosphorous deficiency is initiated through increased renal losses, secondary to increased PTH. As vitamin D is involved in intestinal calcium absorption, a deficient state will lead to low serum calcium levels with a subsequent rise in PTH levels. Decreased levels of phosphorous (PTH stimulates urinary loss) and calcium will result in a reduced calcium\*phosphorous product which negatively affects bone mineralisation and leads to the abnormal organisation of the cartilaginous growth plates, and to hypertrophied chondrocytes (Holick, 1996; Misra et al., 2008). Rickets occurs in three stages following several months of vitamin D deficiency (Wagner & Greer, 2008). The first stage is characterised by osteopaenia and transitory subclinical or overt hypocalcaemia (Misra et al., 2008). In the second stage hypocalcaemia is corrected by rising PTH levels that cause calcium mobilisation from the bone. Bone pain is caused by hydration and swelling of the demineralised collagen matrix. In the final stage, bone changes become more severe, leading to growth retardation, and hypocalcaemia re-emerges. The physical characteristics of rickets include bowed legs or knock knees associated with weight-bearing and a waddling gait; cupping, splaying or fraying at the end of long bones (wrists and ankles); swollen costochondral junctions of the ribs (rachitic rosary) and frontal blossing (Thacher et al., 2002; Wharton & Bishop, 2003).

Rickets was commonly seen in children living in urban industrialised cities throughout Europe at the turn of the 20<sup>th</sup> century, with prevalence increasing during the industrial revolution (Holick, 1994; Thacher et al., 2006). A lack of sunlight was ascertained to be the cause, as children living in rural areas and less-developed countries had a lower incidence of

rickets (Palm, 1980). The use of cod liver oil to prevent and treat rickets resulted in the classification of rickets as a nutritional-deficiency disease (Chick, 1976; Mayer, 1957). Furthermore, the antirachitic discovery of cod liver oil led to the characterisation and synthesis of vitamin D, and subsequent fortification of milk, which effectively eradicated rickets (Holick, 1994; Thacher et al., 2006). In the last 30 years rickets has re-emerged as a global health concern, largely through its identification via published case studies, retrospective chart reviews and cross-sectional studies (Thacher et al., 2006; Weisberg et al., 2004). These reports have come from countries with both limited and plentiful sunshine, including the United States of America (USA), the United Kingdom (UK), Australia, New Zealand, Saudi Arabia, Ethiopia and Canada (Karrar, 1998; Ladhani et al., 2004; Nozza & Rodda, 2001; Robinson et al., 2006; Rowe, 2001; Shaw & Pal, 2002). Several factors have contributed to this, including lack of sunlight exposure, industrial pollution, covering up for religious reasons, poor maternal vitamin D status, prematurity and breastfeeding (Abrams, 2002; Misra et al., 2008; Wagner & Greer, 2008). Increasing immigrant populations in developed countries have also contributed to the resurgence of rickets (Blok et al., 2000; Hintzpetter et al., 2008; Lawson & Thomas, 1999). These populations are at a higher risk for several reasons: the latitude of their new home countries may be higher compared to their country of origin, sun exposure may not be culturally acceptable, food patterns may affect vitamin D and calcium intakes, and many are dark-skinned (Pedersen et al., 2003; Robinson et al., 2006).

Due to this significant increase in the prevalence of rickets in North America (Lazol et al., 2008; Weisberg et al., 2004), the American Academy of Pediatrics recently revised the recommendations regarding vitamin D intake to help ensure adequate vitamin D status (Wagner & Greer, 2008). These recommendations were based on clinical evidence

demonstrating that 400 IU of vitamin D (the amount found in a teaspoon of cod liver oil) both prevented and treated rickets. Although no consensus has been reached with regard to the concentration of 25(OH)D to define vitamin D insufficiency, evidence indicates that daily intake of 400 IU vitamin D results in 25(OH)D concentrations  $> 50$  nmol/L – the concentration attributed to vitamin D sufficiency in adults (Institute of Medicine, 2010). The specific 25(OH)D concentration associated with rickets varies widely from below 30 nmol/L to 50 nmol/L, however (Cranney et al., 2007). In North America, it is now recommended that all infants, children and adolescents obtain at least 400 IU/d of vitamin D beginning in the first few days of life (Wagner & Greer, 2008). For infants exclusively breastfed infants or consuming  $< 1000$  ml/d vitamin D-fortified formula and for adolescents consuming insufficient amounts of vitamin D-rich foods, it is advised that this recommended intake be met through the consumption of a supplement. In New Zealand, it is recommended that infants and pregnant women, especially if they are dark-skinned or veiled, should be screened for vitamin D deficiency and treated accordingly (Munns et al., 2006). To prevent vitamin D deficiency, breastfed infants of dark-skinned or veiled females and at-risk children should receive 400 IU of vitamin D daily. The issue of mandatory, rather than voluntary, fortification of foods such as margarine, milk and dairy products may also need to be considered as a method of preventing rickets (Scragg et al., 2007).

### **Osteomalacia**

Osteomalacia arises from impairment in the mineralisation phase of bone remodelling in the mature skeleton (after the epiphyseal plates close) (Holick, 1996). Osteomalacia is found within the same spectrum as rickets; however where rickets is an entity in which mineralisation is decreased at the level of the growth plates, osteomalacia results in the

undermineralisation of the osteoid bone. As a result the newly formed osteoid, or bone tissue, is soft instead of rigid.

Patients with osteomalacia often have deep bone pain (Eriksen et al., 2002; Plotnikoff & Quigley, 2003). This pain may be due to the improper formation of the collagen-rich bony matrix and resulting hydration and expansion under the periosteal covering (Holick, 2003b). The periosteum contains numerous nociceptors (nerve-endings that initiate pain sensation), accordingly the resulting pressure leads to intense feelings of bone pain (Holick, 2003b). Osteomalacia also results in impaired muscle function and increased risk of fracture due to osteopaenia (bone thinning) (Eriksen et al., 2002; Holick, 1996).

### **Osteoporosis**

Osteoporosis is a multifactorial disease, and its development is influenced by many factors that impact bone remodelling during adult life, as well as bone accrual in early life (Fassler & Bonjour, 1995; Raisz, 2005). Vitamin D insufficiency promotes the development of osteoporosis through the association with PTH. The increase in PTH enhances osteoclast activation; this stimulates an increase in preosteoclast conversion to osteoclasts (Holick, 2006). Osteoclasts resorb bone through the demineralisation of the collagen matrix in bone, leading to osteopaenia, osteoporosis and increased fracture risk (Heaney, 1999; Martin & Seeman, 2008; Raisz, 2005; Sunyecz, 2008). Thus calcium and vitamin D are fundamental contributors to osteoporosis management.

Vitamin D supplementation has been shown to decrease bone turnover and increase bone mineral density (BMD) in randomised controlled trials (RCTs) (Daniele et al., 2004; Dawson-Hughes et al., 1991; Grados et al., 2003; Nordin et al., 1985; Zhu et al., 2008);

however other RCTs have found that vitamin D supplementation increases serum 25(OH)D concentration, but has no overall effect on BMD (Aloia et al., 2005; Andersen et al., 2008). Vitamin D insufficiency has been linked to increased risk of fractures in observational studies, but the most important functional outcome (clinical benefit) is the prevention of osteoporosis-related fractures. Trials assessing the effect of vitamin D supplementation (often with calcium) have produced conflicting results (Chapuy et al., 2002; Grant, 2005; Harwood et al., 2004; Jackson et al., 2006; Larsen et al., 2004; Lyons et al., 2007; Meyer et al., 2002; Nieves et al., 2008; Porthouse et al., 2005; Salovaara et al., 2010; Sanders et al., 2010; Smith et al., 2007). A recent meta-analysis combined the results of 12 double-blind RCTs to determine the effect of vitamin D supplementation on nonvertebral fractures (Bischoff-Ferrari et al., 2009). Supplementation with  $> 400$  IU/d vitamin D reduced nonvertebral fractures by 20% [95% confidence interval (CI) 0.72–0.82], but no effect was seen when supplemental levels were  $\leq 400$  IU/d. There appeared to be an increased benefit of supplementation with vitamin D<sub>3</sub> over vitamin D<sub>2</sub> (23% vs. 10% fracture reduction, respectively). Furthermore, additional calcium did not increase anti-fracture efficacy. An updated Cochrane systematic review of vitamin D evaluated the results of vitamin D supplementation on preventing fractures in the elderly in 45 randomised and quasi-randomised trials (Avenell et al., 2005). They found that vitamin D supplementation alone did not reduce fracture risk, however when vitamin D and calcium supplements were provided there was a 14% reduction in hip fractures (95% CI 0.75–0.96). Wolpowitz and Gilchrist (2006) suggest that the efficacy of vitamin D supplementation in preventing fractures is dependent on baseline vitamin D status and final serum 25(OH)D concentrations.

### **Skeletal muscle strength and falls**

Early clinical reports of myopathy (muscle weakness and hypotonia) associated with severe vitamin D deficiency highlighted a potential role of vitamin D in muscle function (Prineas et al., 1965). The identification of VDR in skeletal muscle tissue has furthered our understanding of the functional role of vitamin D in muscle tissue and physical performance.

The role vitamin D plays in muscle strength has been examined by looking at markers of physical performance such as balance and gait. In observational studies higher 25(OH)D concentrations ( $> 75$  nmol/L) are associated with improved physical performance (Bischoff-Ferrari et al., 2004b; Gerdhem et al., 2005; Shardell et al., 2009; Wicherts et al., 2007). Individuals, 60 years or older, with serum 25(OH)D levels up to 94 nmol/L were able to walk and get out of a chair faster than subjects with 25(OH)D  $< 60$  nmol/L, regardless of whether they were active or sedentary (Bischoff-Ferrari et al., 2004b). Those with lower serum 25(OH)D levels in the Longitudinal Aging Study Amsterdam (LASA) had a greater risk of decreased grip strength and appendicular muscle mass over three years (Visser et al., 2003). Vitamin D supplementation with calcium (vs. calcium alone) improved body sway by 9% in ambulatory elderly women with serum 25(OH)D concentration  $< 50$  nmol/L after 8 weeks (Pfeifer et al., 2000). Lower extremity muscle performance was improved by 4–11%, in institutionalised elderly with 25(OH)D  $< 50$  nmol/L given vitamin D and calcium supplements for 12 weeks, compared to those who received calcium supplements alone (Bischoff et al., 2003). Choice reaction time, postural performance and aggregate functional performance time also significantly improved in the elderly following vitamin D supplementation (Dhesi et al., 2004).

Low serum 25(OH)D concentrations are also associated with increased risk of falling (Sambrook et al., 2004; Snijder et al., 2006; Suzuki et al., 2008). Results from LASA showed that in those older than 75 years of age serum 25(OH)D < 25 nmol/L was associated with an increased risk of repeated falling (Snijder et al., 2006). Treatment with 800 IU vitamin D<sub>3</sub> and 800 mg calcium/d reduced the risk of falling in the elderly by 49%, compared to 800 mg calcium alone (Bischoff et al., 2003). However, results from RCTs have not been consistent, with some trials finding no reduction in falls (Chapuy et al., 2002; Latham et al., 2003). A recent meta-analysis found that supplementation with oral vitamin D alone did not significantly decrease falls, but did in combination with calcium [odds ratio (OR) 0.85; 95% CI 0.76–0.96] (Cranney et al., 2007). Reduction in falls appears to be dose-dependent (Broe et al., 2007), similar to fracture-reduction in the elderly, thus supplementation should be provided at 800 IU/d rather than 400 IU/d. Furthermore, there is a significant decline in VDR expression with age (Bischoff-Ferrari et al., 2004a), suggesting that increased vitamin D is required to help lessen the increase in fall risk that occurs with age.

#### **2.2.4 Implications of low vitamin D status on non-skeletal health**

##### **Cancer**

In the 1980s the first associations between cancer and vitamin D were reported, with those living at higher latitudes in the United States having an increased risk of breast and colon cancer (Garland et al., 1989). Ecologic studies suggest an inverse association between the incidence and mortality from various cancers and solar UVB exposure (Freedman et al., 2002; Grant, 2002a, 2002b, 2004; Luscombe et al., 2001). Case-control, prospective and retrospective observational studies have reported that higher serum 25(OH)D concentrations are associated with lower incidence of breast, colorectal, ovarian and prostate cancers

(Abbas et al., 2008b; Ahonen et al., 2000; Bertone-Johnson et al., 2005; Corder et al., 1993; Feskanich et al., 2004; Hansen, 2001; Jenab et al., 2010; Li et al., 2007; Lowe et al., 2005; Tangrea et al., 1997; Tworoger et al., 2007). However, other prospective and case-control studies have not found a significant relationship between vitamin D status and various cancers (Kampman et al., 2000; Levine et al., 2001; Platz et al., 2004).

There has been little work done to assess the efficacy of vitamin D in preventing cancer, and RCTs are needed to strengthen the evidence provided by epidemiologic studies. A supplementation trial that administered 1100 IU/d vitamin D<sub>3</sub> and 1450 mg/d calcium to post-menopausal women resulted in a 60% reduction in incidence of all invasive cancers (95% CI 0.20–0.82) over four years (Lappe et al., 2007). Calcium intake and vitamin D status may also be interrelated in preventing colon cancer. Another intervention study assessed the effects of supplementation with 400 IU/d vitamin D and/or 500mg/d calcium for six months (Holt et al., 2002). Results indicated that supplementation had no significant effect on cell proliferation in any study group; however cell proliferation decreased as 25(OH)D increased in subjects in the calcium and calcium+vitamin D supplemented groups ( $P = 0.05$ ,  $P = 0.008$  respectively). Lastly, the Calcium Polyp Prevention Trial found that calcium supplementation (1200 mg/d) reduced reoccurrence of colorectal adenomas in patients with serum 25(OH)D concentrations  $> 73$  nmol/L [relative risk (RR) 0.71; 95% CI 0.57–0.89] but not in those with 25(OH)D at or below the median (Grau et al., 2003). Furthermore, serum 25(OH)D concentrations were associated with a reduced risk of adenoma recurrence only in the calcium supplemented group (for every 30 nmol/L increase in 25(OH)D RR = 0.88; 95 % CI 0.77–0.99;  $P$  for interaction = 0.006).

Vitamin D is thought to play a role in chemoprevention through the activation of VDR (present in many cell types). This causes differentiation into normal functioning cells and inhibits proliferation, invasiveness, angiogenesis and metastatic potential of tumour cells (Giovannucci, 2005; Holick, 2006). Vitamin D has also been found to inhibit the growth of cancer cells (Deeb et al., 2007; Kovalenko et al., 2010).

### **Autoimmune disease**

Ecological evidence has linked UV exposure, increasing latitude, and thus vitamin D, to the incidence of autoimmune diseases such as multiple sclerosis (MS), Type 1 diabetes mellitus (T1DM), Crohn's disease and rheumatoid arthritis (Grant, 2006; Holick, 2006; Ponsonby et al., 2005; Wolpowitz & Gilchrest, 2006). Evidence of the role of vitamin D in the regulation of T and B cells, macrophages and dendritic cells continues to accumulate, strengthening the link between vitamin D and the above autoimmune-related diseases (Adorini, 2002; Adorini et al., 2004; Kent et al., 2005; Smolders et al., 2009). However, as with other ecological studies confounding factors cannot be completely controlled for, and there is wide variation among individual UV exposures within a given population.

Type 1 diabetes results from autoimmune destruction of pancreatic  $\beta$  cells, which leads to insulin deficiency (Atkinson & Maclaren, 1994). A gradient of increasing prevalence with higher latitude has been reported for T1DM (Akerblom et al., 1988; Mohr et al., 2008). For example, an investigation of climatic temperature and latitude was found to explain 40% of the variation in T1DM incidence across 15 countries (Akerblom et al., 1988). In Australia, an inverse relationship between annual ambient UV radiation and T1DM prevalence has been demonstrated ( $r = -0.80$ ,  $P = 0.018$ ) and a positive association found at increasing latitude of residence ( $r = 0.70$ ,  $P = 0.046$ ) (Staples et al., 2003). A seasonal pattern of birth

with an increase in incidence in spring and summer has also been reported (Rothwell et al., 1996; Samuelsson et al., 1999), although these findings have been mixed (McKinney, 2001; Rothwell et al., 1999). Vitamin D supplementation or cod liver oil (vitamin D rich oil) appears to be associated with reduced the risk of development of T1DM in childhood. Two case-control studies have reported a reduction in risk of T1DM following supplementation with vitamin D in infancy (Dahlquist, 1999) or cod liver oil during pregnancy (Stene et al., 2000). In a Finnish birth cohort, children were observed for 31 years, following supplementation with 2000 IU/d vitamin D for the first year of life and found to have a 78% reduction in risk of developing T1DM (95% CI 0.05–0.89) (Hyponen et al., 2001). Conversely, Stene et al. (2000) did not find any significant risk reduction in children supplemented during the first year of life, although vitamin D dose was not ascertained. Likewise, supplementation with vitamin D during infancy also had no effect of diabetes-related autoantibodies after 2.5 years (Brekke & Ludvigsson, 2007). The role of vitamin D in diabetes is attributed to the immunomodulating function of 1,25(OH)D, which reduces cytokine production and lymphocyte proliferation. These factors contribute to the destruction of  $\beta$ -cells (that secrete insulin) in the pancreas (Casteels et al., 1998).

Multiple sclerosis (MS) is characterised by central nervous system (CNS) inflammation and demyelination, and is the leading causes of neurologic disability in early to middle adulthood. MS demonstrates a strong gradient of increasing prevalence with higher latitude. For example, benefits of UV exposure with regard to MS onset have been seen in both children and adults (McMichael & Hall, 1997; van der Mei et al., 2001; van der Mei et al., 2003). Season of birth also appears to alter MS risk, with a spring birth excess reported for most (Fernandes et al., 2009; Salzer et al., 2010; Torrey et al., 2000), but not all studies (Sadovnick & Yee, 1994). In a group of women from the Nurses' Health Study,

dietary vitamin D intake was also examined in relation to risk of developing MS (173 cases). Those in the highest quintile of vitamin D intake from food and supplements were less likely to develop MS compared to those in the lowest quintile of intake (RR 0.67; 95% CI 0.40–1.12, *P* for trend = 0.03) (Munger et al., 2004). Supplemental vitamin D intake  $\geq$  400 IU/d was associated with a 40% reduction in risk of MS compared to no supplemental intake (95% CI 0.38–0.91, *P* for trend = 0.006). In a follow-up study from this cohort (*n* = 444), higher serum 25(OH)D levels were associated with a reduced risk of developing MS (OR for a 50 nmol/L increase in 25(OH)D 0.59; 95% CI 0.36–0.97) (Munger et al., 2006). Furthermore, the clinical course of relapsing-remitting MS may be modulated by vitamin D metabolites, through their anti-inflammatory actions. Vitamin D supplementation increased serum transforming growth factor  $\beta$ 1 levels in patients with MS, compared to a placebo (Mahon et al., 2003). Higher levels of transforming growth factor  $\beta$ 1 are seen in the stable phase of MS, whereas lower levels are connected to relapse-remitting MS (Carrieri et al., 1998; Losy & Michalowska-Wender, 2002).

### **Mood disorders**

Several studies have reported an association between suboptimal vitamin D status and a number of mood disorders including pre-menstrual syndrome, seasonal affective disorder (SAD) and other depressive disorders. Both VDR and the enzyme 1- $\alpha$ -hydroxylase are widely distributed throughout the CNS and the brain, with the highest concentrations in the hypothalamus (Eyles et al., 2005), thus it is likely that vitamin D plays a role in neuroendocrine functioning. Mood changes appear to occur with the seasons, an extreme form of which is SAD. This seasonality may be the result of role of vitamin D in the regulation of serotonin (5-HT) (Stumpf & Privette, 1989), as low 5-HT has been linked to the symptoms of SAD, depression and anxiety (Baldwin & Rudge, 1995; Meltzer, 1990).

However, the seasonality of these effects on depressive symptoms may be the result of sunlight itself rather than related to alterations in vitamin D status, caused by reduced sunlight exposure.

The majority of studies comparing mood or depression and vitamin D status are cross-sectional, with lower serum 25(OH)D concentrations associated with higher depression scores (Armstrong et al., 2006; Eskandari et al., 2007; Hoogendijk et al., 2008; Jorde et al., 2008; Jorde et al., 2006). In intervention studies, Jorde et al. (2008) randomised more than 300 overweight subjects to receive either 20 000 IU/wk, 40 000 IU/wk or a placebo for a one year period. There was a significant reduction (improvement) in Beck Depression Inventory scores in both supplemented groups compared to the placebo group ( $P < 0.05$ ). In an earlier supplementation trial, 44 healthy subjects were randomised to 0, 400 and 800 IU/d of vitamin D over five days in winter (Lansdowne & Provost, 1998). The vitamin D supplements enhanced positive affect scores on the Positive and Negative Affect Schedule. However, findings have not been consistent (Dumville et al., 2006; Harris & Dawson-Hughes, 1993). For example, Dumville et al. (2006) assessed seasonal changes in mental health in 1621 elderly women, taking 800 IU/d of vitamin D in a randomised trial. No improvement in mental health scores or quality of life (evaluated by a questionnaire) were observed after six months of intervention (Dumville et al., 2006).

## **2.3 Indices of Vitamin D Status**

### **2.3.1 Serum 25-hydroxyvitamin D**

Serum or plasma 25(OH)D concentration is the best indicator of vitamin D status because it has a long half-life (two to three weeks) (Davie et al., 1982) and reflects the supply of vitamin D from both cutaneous synthesis and dietary sources (Bouillon et al., 1998; Brannon et al., 2008; Davis, 2008; Davis et al., 2007; DeLuca, 2004; Holick, 1994; Norman, 1998). In contrast, circulating 1,25(OH)<sub>2</sub>D concentration should not be used to assess vitamin D status due to its very short half-life (four to six hours) (Brandi et al., 2002) and tight regulation by serum calcium, phosphorous and PTH. Specifically, 1,25(OH)<sub>2</sub>D concentrations do not vary with mild vitamin D deficiency, and often rise and then fall only as vitamin D deficiency becomes more severe (Gibson, 2005; Holick, 2004a; Holick, 2007).

There are several different method principles applied in the measurement of circulating 25(OH)D concentrations that are associated with notable limitations, resulting in marked variation in the estimation of 25(OH)D (Binkley et al., 2004; Carter et al., 2004; Lips et al., 1999). These include both direct detection methods such as high-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) procedures, and indirect detection using competitive protein binding (CPB) and radioimmunoassays (RIAs) (Hollis, 2008b; Hollis & Horst, 2007).

The first assays used to assess circulating 25(OH)D concentrations utilised CPB techniques with DBP as the binder (Holick, 2009). Competitive protein binding assays recognise both 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub> equally, however other vitamin D metabolites

present in serum samples, such as 24,25-dihydroxyvitamin D, 25,26-dihydroxyvitamin D and 25,25-dihydroxyvitamin D-26,23-lactone are also measured (Holick, 2009; Hollis, 2008b). Following this, an RIA was developed that uses an antibody that is co-specific for 25(OH)D<sub>3</sub> and 25(OH)D<sub>2</sub> and incorporates a <sup>125</sup>I-labelled reporter and calibrator (Hollis, 2008b). The RIA also recognises other polar vitamin D metabolites, and consequently both RIA and CPB assays overestimate 25(OH)D levels by 10–20% (Holick, 2009).

Both HPLC and LC-MS methods are able to differentiate between vitamin D<sub>2</sub> and vitamin D<sub>3</sub> in serum (Hollis & Horst, 2007; Maunsell et al., 2005). HPLC with UV detection is generally considered the gold standard method for assessing vitamin D status but it is costly, requires a large sample volume and a radioactive internal standard (Gibson, 2005; Hollis, 2008b). A recent analysis of the accuracy of methodologies used to measure 25(OH)D showed that plasma 25(OH)D<sub>3</sub> levels were not different when measured using LC-MS and HPLC methods, but other methods resulted in significantly different concentrations (Roth et al., 2008). The LC-MS equipment is also expensive, but when properly executed it is an accurate method with higher through put than HPLC (Hollis & Horst, 2007; Maunsell et al., 2005). One disadvantage of LC-MS is that it is unable to discriminate between 25(OH)D<sub>3</sub> and its inactive isomer 3-epi-25(OH)D<sub>3</sub>, a common circulating metabolite in newborn infants (Hollis, 2008b).

Due to concerns regarding the quality and accuracy of the methods used to measure 25(OH)D, the National Institute of Standards and Technology developed a reference sample for vitamin D in blood serum (Chemical Science and Technology Laboratory, 2009). The standard Reference Material 972 can be used to gauge the accuracy of these results.

Although the majority of circulating 25(OH)D is derived from sunlight and from food sources containing vitamin D<sub>3</sub>, supplements and fortified foods may contain vitamin D<sub>2</sub>. If vitamin D<sub>2</sub> is present in the food supply and/or supplements, 25(OH)D levels need to be measured with a method that recognises both forms, to ensure vitamin D status is not being underestimated (Cavalier et al., 2009).

### **Interpretive criteria**

There is currently no universally accepted measure of adequate levels of 25(OH)D (Hollis, 2005; Vieth, 1999). Variations in methods used to assess 25(OH)D concentrations coupled with the limitations of these methods make it difficult to draw conclusions when defining cut-offs for hypovitaminosis D. It is inappropriate to use population-based reference ranges for 25(OH)D to determine status, as these are dependent on diet and UV exposure and vary greatly depending on ethnicity and latitude, among other factors. To date, the establishment of adequate vitamin D status has focused on the level of circulating 25(OH)D concentrations needed to support bone remodelling (Hollis, 2008a). Consequently, vitamin D deficiency is defined as the presence of signs/symptoms that characterise skeletal disease states (i.e. rickets and osteomalacia), typically occurring at circulating 25(OH)D concentrations of less than 30 nmol/L (12 ng/ml) (Institute of Medicine, 2010; Misra et al., 2008) (**Table 2.2**). However, the concentration at which rickets occurs is highly variable, with radiologic changes demonstrated in infants with 25(OH)D concentrations up to 40–45 nmol/L (Kreiter et al., 2000). Rickets is primarily caused by low vitamin D status, though inadequate dietary calcium intake may play a significant role (Wilson, 2005). The evidence that adverse outcomes such as poor muscle function, low bone mineral density and fracture risk occur at higher 25(OH)D concentrations has led to proposals to increase the vitamin D deficiency cut-offs (Prentice et al., 2008; Wolpowitz & Gilchrest, 2006).

**Table 2.2: Biochemical signs of different stages of vitamin D deficiency<sup>1</sup>**

	Plasma calcium	Plasma phosphate	Alkaline phosphatase	PTH	25(OH)D	1,25(OH) <sub>2</sub> D	Radiograph changes
Early	N <sup>2</sup> / ↓	N / ↓	↑	↑	↓	N	Osteopaenia
Moderate	N / ↓	↓	↑↑	↑↑	↓↓		Rachitic changes +
Severe	↓↓	↓↓	↑↑↑	↑↑↑	↓↓↓	↑ / N / ↓	Rachitic changes ++

<sup>1</sup> Originally adapted from Kappy MS, Allen DB, Geffner ME, eds. Principles and Practice of Pediatric Endocrinology (2005). Springfield, Charles C Thomas Co.; 762 in Misra et al. 2008 (Misra et al., 2008)

<sup>2</sup>N indicates normal; ↓, decrease; ↑, increase; +, mild changes; ++, moderate to severe changes

Sufficiency refers to a concentration of 25(OH)D where there are no abnormalities in calcium homeostasis (Wolpowitz & Gilcrest, 2006). A range of 25(OH)D concentrations have been proposed to be sufficient including > 50 nmol/L (Lips, 2001; Norman et al., 2007; Ross et al., 2010) and > 75 nmol/L (Durazo-Arvizu et al., 2010; Vieth et al., 2007). Insufficiency is a term used to describe plasma 25(OH)D levels between absolute deficiency (rickets/osteomalacia) and the level of sufficiency (Heaney, 2004). The range of insufficiency has not been clearly defined. It has been proposed that insufficient vitamin D levels that may be associated with adverse health outcomes (such as malignancies and autoimmune disorders) in the absence of overt clinical disease (Heaney, 2004; Wolpowitz & Gilcrest, 2006). In general, the most often used value for sufficient serum 25(OH)D concentration is > 50 nmol/L in adults (Diamond, 2005; Institute of Medicine, 2010).

As a fat-soluble vitamin, excessive consumption of vitamin D results in excessive absorption, potentially leading to vitamin D toxicity. Vitamin D intoxication results in hypercalcaemia, hypercalciuria and hyperphosphataemia, indicative of excessive

1,25(OH)<sub>2</sub>D activity (Adams & Lee, 1997; Chesney, 1989; Vieth, 1990). Symptoms include acute renal failure and nephrocalcinosis. The maximum normal circulating level of 25(OH)D is about 125 nmol/L, but excessive vitamin D intakes produce concentrations of  $\geq 2500$  nmol/L (Horst et al., 2005). At these concentrations 25(OH)D competes with 1,25(OH)<sub>2</sub>D for binding to VDR, producing actions normally attributed to 1,25(OH)<sub>2</sub>D. Thus signs of vitamin D toxicity occur, despite normal or slightly below normal 1,25(OH)<sub>2</sub>D concentrations.

### **2.3.2 Functional indicators of vitamin D status**

#### **Parathyroid hormone**

The concentration of PTH has been proposed as a functional indicator of the availability of active vitamin D because of its role in the conversion of 25(OH)D to 1,25(OH)<sub>2</sub>D through the calcium-phosphate homeostatic system (Prentice et al., 2008). Parathyroid hormone can be measured from serum or plasma using RIA (Gibson, 2005). An inverse relationship between PTH and 25(OH)D concentrations has been described in a number of different population groups (Bates et al., 2003; Chapuy et al., 1997; Guillemant et al., 1999; Harinarayan, 2005; Lappe et al., 2006). The relationship between PTH and serum 25(OH)D is not completely linear as PTH concentrations eventually plateau once 25(OH)D reaches a certain concentration (ranging from 25 to 125 nmol/L) (Dawson-Hughes et al., 2005; Holick et al., 2005; Vieth et al., 2003). Many have suggested using the threshold of 25(OH)D needed to maximally suppress PTH as a possible cut-off for determining optimal vitamin D status (Durazo-Arvizu et al., 2010). This value threshold varies depending on the study population, their dietary calcium intake, the methods used to measure 25(OH)D and plasma PTH concentration, and differences related to the statistical approach used to model the maximum suppression (Bates et al., 2003; Durazo-Arvizu et al., 2010; Prentice et al.,

2008). Furthermore this cut-point appears to be dependent on calcium intake. It has been noted that for those with average calcium intakes 700–1000 mg/day, the 25(OH)D-threshold concentration was reached at 75–80 nmol/L (Aloia et al., 2006; Dawson-Hughes et al., 2005). Provided calcium intake is sufficient, an elevated PTH in conjunction with a low 25(OH)D concentration provides a good indication of vitamin D insufficiency.

In contrast other studies did not find a cut-point where PTH concentration plateaus, despite showing a significant inverse relationship between PTH and 25(OH)D (Bates et al., 2003; Benjamin et al., 2009; Guillemant & Guillemant, 1996; Vieth et al., 2003). On this basis some authors have rejected the clinical utility of the threshold as a way of identifying optimal vitamin D status (Aloia et al., 2006; Heaney, 2005; Vieth & Fuleihan, 2005). It has been noted by several experts that the mathematical approach that has been used to establish a PTH concentration plateau in relation to 25(OH)D concentration is biased in that it assumes such a plateau exists (Chapuy et al., 1997; Vieth & Fuleihan, 2005). The use of a locally weighted scatterplot smoother line (LOWESS) removes researcher driven bias, and when Vieth and Fuleihan (2005) plotted LOWESS regression lines for log PTH vs. log 25(OH)D concentrations from 1858 subjects no plateau was observed. Thus a target level for 25(OH)D may not be able to be obtained from PTH concentrations. Moreover, at very high 25(OH)D concentrations an increase in PTH has been shown, which may be indicative of poor renal function (Bates et al., 2003). In order to rationalise the contrasting results between studies, Durazo-Arvizu et al. (2010) compared thresholds of 25(OH)D and PTH from several 2- and 3-phase regression models that use different assumptions about the shape of the underlying curves and the definition of maximal suppression in a group of healthy adults (n = 387, aged 65–87 yrs). Using the three-phase model, two thresholds of 30 nmol/L and 70 nmol/L were found; PTH concentrations decline at a rapid rate with increasing 25(OH)D

concentrations until the first threshold is reached. After this threshold the rate slows until the second threshold is reached, following this PTH is maximally suppressed. The curve of this three-phase approach is very similar to derived LOWESS curves, but can also be used to simultaneously estimate both thresholds and evaluate the magnitude of the rate of change between thresholds (Durazo-Arvizu et al., 2010).

### **Intestinal calcium absorption**

Another method of functionally evaluating the optimal level of 25(OH)D is to assess intestinal calcium absorption fraction using stable isotope methods. One of the calciotropic roles performed by 1,25(OH)<sub>2</sub>D involves the active transport of calcium across the intestinal mucosa (DeLuca, 2004; Heaney, 2007). Under conditions of adequate calcium intake, absorptive efficacy increases as serum 25(OH)D increases up to approximately 80 nmol/L (32 ng/ml). Above this threshold additional increases in 25(OH)D status have no further effect on absorptive regulation (Heaney, 2007; Heaney et al., 2003b). At low calcium intakes net calcium absorption is low, irrespective of vitamin D status. Thus, both vitamin D and calcium are required to ensure sufficient net absorption of calcium.

### **Bone turnover markers and bone mineral density**

Biomarkers and functional endpoints, such as bone resorption and BMD, have been suggested to help further define adequate levels of circulating 25(OH)D concentrations, due to the role of vitamin D in bone metabolism and calcium homeostasis. Bone turnover markers include serum osteocalcin, serum bone-specific alkaline phosphatase, serum C-terminal telopeptide of type I collagen, urinary cross-linked *N*-telopeptides of type I collagen and urinary deoxypyridinoline. A systematic review of six RCTs that assessed the effect of vitamin D supplementation on markers of bone turnover did not find any of these to be

adequate functional markers of vitamin D status (Seamans & Cashman, 2009). However, it was noted that there were insufficient studies to carry out further subgrouping and that the high heterogeneity of studies due to variable population groups, doses and supplemental form (D<sub>2</sub> or D<sub>3</sub>, additional calcium), limited the analysis for many of the markers investigated. High inter-subject variability and other limitations associated with biochemical markers makes the assessment of bone turnover markers challenging (Hannon & Eastell, 2000). Since the time of that review, results from subsequent RCTs undertaken in adolescents, young adults and the elderly found that vitamin D supplementation does not alter markers of bone turnover (Molgaard et al., 2010; Seamans et al., 2010). There were also no significant interactions between supplementation groups and VDR and gene polymorphisms.

The effect of vitamin D supplementation on BMD in RCTs has also been assessed; as a result of the fundamental effect of vitamin D is maintenance of a healthy skeleton. In the elderly, a systematic review assessing the effect of vitamin D on 25(OH)D and BMD indicated that supplementation increased whole-body BMD significantly with no heterogeneity (weighted mean difference (WMD): 0.10%; 95% CI 0.03–0.17) and increased lumbar spine BMD significantly with no heterogeneity (WMD: 0.82%; 95% CI 0.60–0.83) (Seamans & Cashman, 2009). However, an RCT assessing the combined effects of calcium (1000 mg/d)-vitamin D<sub>3</sub> (800 IU/d) and exercise, in 180 elderly men found that exercise increased BMD, but additional calcium-vitamin D supplements did not enhance the osteogenic response (Kukuljan et al., 2011). Studies in adolescents and children have produced mixed results (Ala-Houhala et al., 1988; Benchimol et al., 2007; Du et al., 2004; El-Hajj Fuleihan et al., 2006; Molgaard et al., 2010; Viljakainen et al., 2006a). Consumption of vitamin D<sub>3</sub> and calcium fortified milk for two years significantly increased bone mineral

density compared to consumption of milk fortified with calcium alone (5.5% vs. 3.2%) in girls aged 10 years (n = 757) (Du et al., 2004). Vitamin D<sub>2</sub> supplementation in either children with irritable bowel disease or healthy pre-pubertal children did not affect BMD (Ala-Houhala et al., 1988; Benchimol et al., 2007). The effect on BMD following administration of either 200 or 400 IU vitamin D<sub>3</sub>/d for one year to girls aged 11–12 years, was also not significant (Molgaard et al., 2010).

### **2.3.3 Factors affecting serum 25-hydroxyvitamin D concentrations**

There are several factors that affect photochemical regulation of previtamin D<sub>3</sub> production, including environmental, physiological and behavioural factors.

#### **Season, latitude and time of day**

The angle that sunlight passes through the earth's atmosphere has an effect on the amount of UVB light that is absorbed by the ozone (Holick, 2004b; Prentice, 2008). As latitude increases, the zenith angle of the sun alters causing increased absorption of UVB photons, which reduces the potential for vitamin D synthesis. The angle of the sun also changes according to season and time of day. At lower latitudes the angle of the sun is acute, thus exposure of the skin to sunlight induces cutaneous synthesis of vitamin D<sub>3</sub> throughout the year. At latitudes above 37°N and below 37°S, there is not enough sunlight for cutaneous vitamin D synthesis to occur during the winter months (Chen, 1999; Norman, 1998; Webb et al., 1988). Vitamin D status is therefore lower at the end of winter than in summer (Berry et al., 2009; Gozdzik et al., 2008; Kull et al., 2009b; Oliveri et al., 1994; Rapuri et al., 2002; Rockell et al., 2005). The zenith angle is also more oblique early in the morning and late in the afternoon limiting the cutaneous production of vitamin D at these times, even during summer months (Webb et al., 1988).

## **Sun exposure**

Duration and frequency of skin exposure to sunlight has a significant impact on vitamin D status. Sun exposure can be affected by personal factors, such as wearing clothing (for religious/cultural or climatic reasons), sunscreen use and frequency of outdoor activity or being confined indoors (homebound or institutionalised). Sunscreen acts by decreasing the number of UVB photons that reach 7-dehydrocholesterol in the skin, thus chronic sunscreen use results in significantly lower vitamin D levels (Macdonald et al., 2011; Matsuoka et al., 1988; Nicolaidou et al., 2006). Both glass and most plastics absorb UVB radiation, thus sunlight exposure through windows or plexiglass do not result in cutaneous production of vitamin D (Holick, 1994). Clothing also prevents absorption of UVB photons and can have a significant effect on vitamin D status in populations where full-body clothing is deemed culturally appropriate. For example, vitamin D deficiency, rickets and osteomalacia in Saudi Arabian children and women have been attributed to cultural dress, which results in very little skin exposure to sunlight (Al-Turki et al., 2008; Grover & Morley, 2001; Sedrani, 1984; Serenius et al., 1984; Taha et al., 1984).

Vitamin D status can also be reduced by environmental factors such as cloud cover, atmospheric pollution and altitude. Indian children living in areas with greater atmospheric pollution had significantly lower serum 25(OH)D concentrations than those living in less polluted areas (31.0 vs. 67.8 nmol/L,  $P < 0.001$ ) (Agarwal et al., 2002). The morbidity rate of rickets in children in Shanghai has also been associated with atmospheric pollution and urban living (Gong et al., 2007).

### **Ethnicity and skin pigmentation**

Melanin competes with 7-dehydrocholesterol for ultraviolet photons. Thus, increased melanin pigmentation reduces the efficiency of UVB conversion of 7-dehydrocholesterol to previtamin D<sub>3</sub> (Holick, 1981). The time of exposure necessary to maximise the formation of previtamin D<sub>3</sub> increases as melanin content of skin increases, thus darker skinned people require a longer period of UV exposure to produce an equivalent amount of vitamin D than fairer skinned people (Matsuoka et al., 1991). It has been reported that an African American requires six times the exposure dose required by a white person to raise circulating vitamin D concentrations equivalently (Clemens et al., 1982). In New Zealand, Maori and Pacific people have lower serum 25(OH)D concentrations than Europeans (Rockell et al., 2005; Rockell et al., 2006). Similarly non-Hispanic blacks (African Americans) have poorer vitamin D status compared to non-Hispanic whites (Caucasians) in the United States (Hall et al., 2010; Looker et al., 2002; Rajakumar et al., 2011b; Stein et al., 2006; Yetley, 2008).

While ethnicity is a proxy for skin colour, wide variations in skin colour are present. Skin pigmentation can be measured using reflectance colourimetry (Individual Typology Angle, ITA). Using skin colour assessment, higher baseline 25(OH)D concentrations have been shown to be correlated with lighter skin as compared to darker skin (Armas et al., 2007). Furthermore, facultative (sun exposed/tanned) skin colour has shown to be a significant predictor of 25(OH)D concentration in Pacific and European people living in New Zealand, whereby a 10° higher ITA (less tanning) was associated with a 5% lower 25(OH)D concentration ( $P = 0.001$ ) (Rockell et al., 2008b). This suggests that tanning is an indicator of sunlight exposure, and subsequently predicts vitamin D status. The increase in 25(OH)D levels following UVB exposure has also been found to be inversely correlated with skin pigmentation (Bogh et al., 2010).

## **Age**

Vitamin D status declines with age, increasing the prevalence of vitamin D deficiency in the elderly (Atli et al., 2005; Papapetrou et al., 2007; Rockell et al., 2006). This may occur as a result of several factors including an age-dependent decrease in dermal 7-dehydrocholesterol concentrations, reduced mobility and greater likelihood of being institutionalised, decreased renal production of 1,25(OH)<sub>2</sub>D and reduced consumption of fortified foods (Bell, 1995; Gloth et al., 1995; MacLaughlin & Holick, 1985; Need et al., 1993; Oudshoorn et al., 2009; Tzotzas et al., 2010).

## **Adiposity**

Adiposity, determined by body mass index (BMI) or body fat content (measured by dual-energy x-ray absorptiometry), is negatively correlated with serum 25(OH)D concentrations, and positively correlated with PTH levels (Arunabh et al., 2003; Grethen et al., 2011; Parikh et al., 2004; Rajakumar et al., 2011a; Snijder et al., 2005; Wortsman et al., 2000). Consequently obese individuals have lower vitamin D levels compared with individuals who are normal weight. This may be due to increased sequestration of this vitamin into subcutaneous adipose tissue (as obese persons have more body fat) or decreased sun exposure (Kull et al., 2009a; Liel et al., 1988; Looker, 2007). Serum 25(OH)D levels also rise when obese individuals lose body fat, supporting the hypothesis that 25(OH)D is stored in adipose tissue and not readily released unless these stores are broken down (Mason et al., 2011; Riedt et al., 2005; Zittermann et al., 2009).

It does not appear that 7-dehydrocholesterol concentrations in the epidermis differ between obese and non-obese persons, nor does its percentage conversion to previtamin D<sub>3</sub>

and vitamin D<sub>3</sub> (MacLaughlin & Holick, 1985; Need et al., 1993). The absorption of dietary vitamin D<sub>2</sub> has also been assessed to determine if there are differences in absorption between obese and non-obese individuals, which may account for the differences in vitamin D status. Wortsman et al. (2000) supplemented 11 obese and 11 non-obese participants with a 50 000 IU loading dose of vitamin D<sub>2</sub>, with no significant group-by-time interaction observed 24 hours after administration. In addition, adipose tissue did not influence the response to supplementation with 7000 IU/wk vitamin D<sub>3</sub> for 12 weeks, in elderly homebound individuals (Canto-Costa et al., 2006).

Differences in willingness to expose skin to sunlight may account for a small proportion of the differences in vitamin D status between obese and normal weight individuals. A recent study found that those with a BMI >30 (kg/m<sup>2</sup>) were more likely to avoid sunbathing and less willing to expose their body to the sun in summer, than those with a BMI < 30 ( $P < 0.01$ ) (Kull et al., 2009a). Obese individuals may also have reduced sun exposure due to avoidance of outdoor activity or limited mobility (Compston et al., 1981).

### **Vitamin D binding protein**

Vitamin D-binding protein (DBP, also known as Gc-globulin) is a multifunctional, polymorphic serum  $\alpha_2$ -globulin protein that is mainly synthesised in the liver (White & Cooke, 2000). It consists of 458 amino acids, with a 16 amino acid leader sequence which is cleaved from the protein prior to secretion (Speeckaert et al., 2006; White & Cooke, 2000). The DBP-gene is contained on the long arm of chromosome 4 (4q12-q13), extending over 35 kb DNA. The most important function of DBP is binding and transporting vitamin D and its metabolites (Daiger et al., 1975). Plasma DBP concentration is much higher than the total amount of vitamin D and its metabolites (Liang & Cooke, 2005), thus under normal

physiological conditions nearly all circulating vitamin D compounds are protein bound (White & Cooke, 2000). As a result, the DBP-vitamin D metabolite complex is less susceptible to hepatic metabolism, prolonging their half-life in circulation. Other biological functions of DBP relate to its role in the immune system and host defence, such as actin scavenging and macrophage activation and chemotaxis (Speeckaert et al., 2006).

Human DBP is a highly polymorphic protein, with three well-known alleles: Gc1S, Gc1F, Gc2 (Constans & Viau, 1977) (**Table 2.3**) and more than 120 identified rare variant alleles (Cleve & Constans, 1988). Two main single nucleotide polymorphisms (SNPs), located on the exons of the DBP gene are the rs7041 SNP (at position 416), which contains a T-to-G transversion resulting in substitution of glutamic acid for aspartic acid, and the rs4588 SNP (at position 420) has a C-to-A transversion (threonine to lysine) (Sinotte et al., 2009). Gc1S has the T-to-G transversion, Gc2 has the C-to-A transversion and Gc1F has neither (Arnaud & Constans, 1993; Braun et al., 1992).

**Table 2.3: Vitamin D binding protein genetic polymorphisms**

<b>Polymorphism</b>	<b>rs7041 (position 416)</b>	<b>rs4588 (position 420)</b>
Gc1S	Glutamic acid	Threonine
Gc1F	Aspartic acid	Threonine
Gc2	Aspartic acid	Lysine

Gc1S and Gc1F exhibit different worldwide geographical distributions and racial variations (Constans et al., 1985; Speeckaert et al., 2006). Populations with white skin have lower Gc1F-allele and higher Gc1S-allele frequencies, whereas the Gc1F-allele is markedly higher in black Africans and black Americans. These allele frequencies display a typical geographical cline from Southeast Asia, through Europe and the Middle East, and down to

Africa (Speeckaert et al., 2006). All populations appear to have a lower prevalence of the Gc2-allele compared to that of the Gc1-allele. Nevertheless, Caucasians have a noticeably higher occurrence of the Gc2-allele compared to black populations (Speeckaert et al., 2006).

GC variants of DBP differ in their affinity for vitamin D metabolites, and thus recent studies have investigated the influence of DBP polymorphisms on circulating levels of 25(OH)D. Indeed, several studies have demonstrated that the Gc2 phenotype allele results in lower circulating 25(OH)D and DBP levels, compared to the Gc1 (Gc1S and Gc1F) alleles (Abbas et al., 2008a; Arnaud & Constans, 1993; Lauridsen et al., 2005; Lauridsen et al., 2001). However, other studies have found no relationship between DBP and 25(OH)D level (Taes et al., 2006; Winters et al., 2009). Regardless of ethnicity, the lysine substitution in Gc2 was associated with lower levels of 25(OH)D and 1,25(OH)<sub>2</sub>D compared to the Gc1 proteins (Engelman et al., 2008). Furthermore, the glutamic acid substitution in Gc1S was associated with a higher 25(OH)D concentration. Sinotte et al. (2009) also found that both substitutions were associated with lower adjusted mean  $\pm$  SE 25(OH)D concentrations; aspartic acid (at 416)  $60.8 \pm 1.5$  vs. glutamic acid  $67.5 \pm 1.1$  nmol/L ( $P < 0.001$ ) and lysine (at 420)  $58.4 \pm 2.0$  vs. threonine  $67.2 \pm 0.9$  nmol/L ( $P < 0.001$ ).

The observed increase in 25(OH)D following vitamin D<sub>3</sub> supplementation also appears to be partly allele-specific. The response of 25(OH)D to supplementation was examined in a group of healthy adults given two doses of vitamin D (600 or 4000 IU) (Fu et al., 2009). The percentage increase following supplementation was significantly affected by the rs4588 genotype (Gc2), in contrast to the rs4071 (Gc1F) genotype which did not significantly affect the increase following supplementation. Examination of baseline 25(OH)D concentrations suggested a trend toward lower values in participants homozygous for the Gc2-allele. These

participants had the greatest proportional increase after supplementation (307%), followed by the heterozygous Gc2 group (151%) and participants with Gc1 alleles (97%) ( $P = 0.004$ ) (Fu et al., 2009).

### **Other factors**

Those who do not regularly consume vitamin D-rich or fortified foods are likely to have lower vitamin D status (Holick, 2006). Vitamin D is only readily available in few foods thus is not easily obtained from a regular diet. Dietary vitamin D is absorbed through the lipid pathway, accordingly patients with fat malabsorption syndromes such as Cystic Fibrosis and Crohn's disease are also at risk of vitamin D deficiency and have lower levels of 25(OH)D (Koutkia et al., 2001; Lo et al., 1985). Use of ethinyl oestradiol containing oral contraceptive pills increases serum 25(OH)D concentrations (Harris & Dawson-Hughes, 1998; Nesby-O'Dell et al., 2002). Oestrogen may mediate this effect by increasing DBP concentration, altering the relative proportion of free and protein bound 25(OH)D (Aarskog et al., 1983; Kleerekoper et al., 1991). Anticonvulsant drugs are conversely associated with decreases in 25(OH)D concentrations (Khanna et al., 2009; Mikati et al., 2006).

## **2.4 Vitamin D Requirements in Healthy Adults**

### **2.4.1 Recommended dietary vitamin D intakes**

Several countries and constitutional bodies have published recommendations for dietary vitamin D intake, of which the majority have been established based on the functional indicator of serum 25(OH)D. Most recommend different intake levels for various life-stage groups, including pregnant and lactating women. However, many uncertainties remained when the recommendations were published, including the concentration of 25(OH)D that constitutes the lower limit of adequacy, the amount of vitamin D needed daily to meet and sustain any given level of 25(OH)D and the amount of required input that is derived from endogenous synthesis. As a result of these uncertainties, Adequate Intake (AI) values were set in New Zealand/Australia. Late in 2010, the United States and Canada updated AIs for vitamin D, setting Dietary Reference Intakes (DRIs) in the form of Estimated Average Requirements (EARs) and Recommended Dietary Allowances (RDAs) for all life stages, with the exception of infants aged 12 months or less (Institute of Medicine, 2010). The following principal questions form the basis of the recommended intake decision-making framework (Institute of Medicine, 2010): (1) What is the effect of circulating concentrations of 25(OH)D on health outcomes?; (2) What is the effect of vitamin D intake on circulating concentrations of 25(OH)D?; (3) What is the effect of vitamin D intakes on health outcomes?; and (4) What levels of vitamin D are associated with adverse effects?

## **New Zealand and Australia**

For infants and toddlers aged 0 to 3 years the AI is set at 200 IU/d (5 µg/d) (Australian Government: Department of Health and Aging; National Health and Medical Research Council & Ministry of Health, 2006) based on the lowest dietary intake of vitamin D associated with a mean serum 25(OH)D concentration of > 27.5 nmol/L (lower limit of normal) assuming minimal sunlight exposure (Standing Committee on the Scientific Evaluation of Dietary Reference Intakes et al., 1997). This is the same as the AI for children, adolescents, adults aged 19 to 50 years, pregnant and breastfeeding women, and is also based on the amount of vitamin D required to maintain a serum 25(OH)D concentration > 27.5nmol/L. For older adults, the levels have been set higher: 400 IU/d (10 µg/d) for adults 51 to 70 years of age and 600IU/d (15 µg/d) for adults > 70 years (Australian Government: Department of Health and Aging; National Health and Medical Research Council & Ministry of Health, 2006). The requirement for older adults takes into consideration the reduced capacity for cutaneous vitamin D synthesis and the evidence regarding vitamin D supplementation and bone loss.

The upper level of intake (UL) for Australians and New Zealanders has been set at 1000 IU/d (25 µg) for infants < 12 months of age, and 3200 IU/d (80 µg) for the remainder of the population (Australian Government: Department of Health and Aging; National Health and Medical Research Council & Ministry of Health, 2006). For adults this level was developed based on an uncertainty factor of 1.2 and the established No Observed Adverse Effects Level of 4000 IU/d (100 µg) (derived from an RCT conducted by Vieth et al. (2001) where adverse effects were not seen in 30 adults following supplementation with 4000 IU/d for six months).

## **United States and Canada**

The Institute of Medicine committee recently released an update regarding the requirements for calcium and vitamin D, which emphasises the need for an increased vitamin D intake and encourages its use as a fortificant (Institute of Medicine, 2010). The committee determined that a serum 25(OH)D concentration of 40 nmol/L was consistent with the nature of an average requirement (population median), serum levels of 50 nmol/L were sufficient for the majority of the population and levels > 75 nmol/L were not associated with an additional benefit (Institute of Medicine, 2010). Thus, maintaining concentrations between 40 and 50 nmol/L is desirable. Based on this report, current DRIs have been set assuming no exogenous source of vitamin D from sunlight exposure, to account for diminished cutaneous synthesis in those who live at Northern latitudes year round and Southern latitudes in winter. For infants aged 0–12 months the AI is 400 IU/d (10 µg/d). Intakes above this level should result in 25(OH)D levels above those associated with rickets. For children and adults aged  $\geq 1$  year, the EAR has been set at 400 IU/d (10 µg/d), with no difference for pregnant and lactating women. The RDI is 600 IU (15 µg/d) for those aged 1-70 years and for those older than 70 years it is 800 IU/d (20 µg/d), on account of limited sun exposure and decreased 7-dehydrocholesterol concentrations in the epidermis (Institute of Medicine, 2010).

## **United Kingdom**

Recommended nutrient intakes (RNIs) were established for infants and the elderly in the UK in 1991, however for those aged 4 to 64 years cutaneous vitamin D synthesis is thought to be adequate, so no RNI was set for this population group by the UK Committee on Medical Aspects of Food and Nutrition Policy (Cashman et al., 2008). The RNI for

infants 0–6 months is 340 IU/d and is 280 IU/d for those 6 months to 3 years. For the elderly (> 65 years) the RNI is 400 IU/d (Prentice et al., 1998).

## **2.4.2 Nutritional status of healthy adults**

### **New Zealand and Australia**

There is currently no population representative intake data for vitamin D for New Zealanders. Dietary intakes of vitamin D are low, the mean intakes of adult Australian men and women are 80–120 IU/d, reported in the CSIRO National Dietary Survey and the highest decile of intake was 222 IU/day, in men aged 18–29 years (Nowson & Margerison, 2002). Estimates of vitamin D intakes of new Zealanders obtained using the food additive modelling system are comparable, 80–96 IU/d (Australia New Zealand Food Authority, 1999), despite differences in fortification policies. Current levels of food fortification are likely to have minimal impact on vitamin D status, however it is unlikely that increasing the amount or range of food fortified with vitamin D would rectify deficiencies in high risk groups, but may maintain vitamin D status in low risk groups (Nowson & Margerison, 2002).

Data from the 1997 New Zealand National Nutrition Survey and the 2002 Children's Nutrition Survey indicate that New Zealanders have a high level of vitamin D inadequacy (Rockell et al., 2005; Rockell et al., 2006). Mean serum 25(OH)D concentrations of New Zealand adults and children were 47 and 52 nmol/L, for males and females respectively (Rockell et al., 2005; Rockell et al., 2006). Furthermore, 50% of adults had serum 25(OH)D concentrations < 50 nmol/L, with rates of insufficiency higher in Maori and Pacific people than in Europeans, and higher in winter vs. summer months (Rockell et al., 2006). Parathyroid hormone concentrations as determined in a convenience sample of New Zealand

adults were also higher in early spring vs. summer, reflecting the lower 25(OH)D levels (Rockell et al., 2008a).

Rates of insufficiency (25(OH)D <50 nmol/L) in younger Australian adults are estimated to fall between 23% to 43%, and are 76% in elderly nursing home residents (McGrath et al., 2001; Nowson et al., 2000; Pasco et al., 2001). In another study, 80% of dark-skinned, veiled, pregnant Australian women had frank vitamin D deficiency, with serum 25(OH)D concentrations < 25 nmol/L (Pasco et al., 2001).

The New Zealand Ministry of Health recommends that most adults have a small amount of daily sun exposure, rather than consume supplements to ensure their vitamin D status is adequate. Though, it is recognised that the elderly or institutionalised individuals may need supplements (Ministry of Health, 2003). Routine supplementation is not recommended for infants and women who are pregnant or breastfeeding either, yet those at risk of vitamin D deficiency (have darker pigmented or covered skin) may need to take a supplement under the supervision of a healthcare practitioner (Ministry of Health, 2008).

### **United States of America**

Food consumption data from the National Health and Nutrition Examination Survey (NHANES) 2005–2006 found that children aged eight years or less consumed 260 IU/d vitamin D from food, males aged nine years and older consumed 220 IU/d and females older than eight years of age had a mean intake of 170 IU/d (Bailey et al., 2010). Furthermore vitamin D intakes from food and supplements were significantly higher than from food alone. Previous NHANES surveys found that the use of vitamin D-containing supplements increased dietary vitamin D intakes by 10 to 25% (Moore et al., 2004). Vitamin D intakes

are lowest in the elderly with less than 10% of adults aged 51–70 years and less than 2% of those 70 years and older, determined to be meeting the AI for vitamin D from food alone (Bailey et al., 2010).

Data from the NHANES survey indicated a decline in 25(OH)D concentration of 75 nmol/L in the US population from 1988–1994 to 60 nmol/L in 2000–2004 (Ginde et al., 2009). Rates of insufficiency (< 50nmol/L) also increased from 22% in 1988–1994 to 36% in 2000–2004. Five percent of NHANES 2000–2004 participants also had serum 25(OH)D concentrations < 27.5 nmol/L (11 ng/ml) (Yetley, 2008). Adjusted mean 25(OH)D concentrations decreased with increasing age in adults participating in the NHANES III, with 79, 73 and 63 nmol/L for persons aged 20–39, 40–59 and 60 or more years (Scragg et al., 2008). Vitamin D levels also varied according to ethnicity, being highest in non-Hispanic whites, were inversely associated with BMI, and displayed seasonal variation (March–April, 67 nmol/L vs. July–October, 80 nmol/L) (Scragg et al., 2008). Mean 25(OH)D levels are likely an underestimate as NHANES samples were collected at higher latitudes during the summer and lower latitudes during the winter.

### **Europe, the Middle East and Asia**

Due to differences in sun exposure and dietary and supplemental vitamin D intake, vitamin D status varies greatly amongst the different countries in Europe, the Middle East and Asia (Lips, 2007). In Europe the prevalence of vitamin D deficiency ranges from 2% to 30% in adults, and up to 80% in institutionalised elderly (Lips, 2001). In France mean dietary vitamin D intakes were 160 IU/d for men and 112 IU/d for women. Serum 25(OH)D levels were higher in the south west of France (94 nmol/L) compared to the north (43 nmol/L), and were positively correlated with sun exposure and negatively correlated with

latitude (Chapuy et al., 1997). Conversely, elderly Italian and Spanish adults had lower 25(OH)D concentrations, with a higher prevalence of deficiency, than adults in Norway, Sweden and Finland (Lips, 2007; Van der Wielen et al., 1995). This may be indicative of a greater influence of supplement use and food fortification rather than increased UVB exposure and its influence on vitamin D status.

Studies from Middle Eastern countries indicated that vitamin D status is influenced by clothing rather than sun exposure. Women who wore Western style clothing had better vitamin D status than women who wore veils, and in general, vitamin D status was lower in women compared to men (Lips, 2007). There is little information available on dietary vitamin D intakes, but estimates range from 76 IU/d to 100IU/d (Gannage-Yared et al., 2000; Meddeb et al., 2005).

In Asian countries, latitude influences vitamin D status, the prevalence of low vitamin D status ( $25(\text{OH})\text{D} < 25 \text{ nmol/L}$ ) was 47% in Asian Russian ( $57^\circ\text{N}$ ) adults compared to 5% of Japanese ( $36^\circ\text{N}$ ) women and 2% of Malaysian ( $30^\circ\text{N}$ ) women (Bakhtiyarova et al., 2005; Rahman et al., 2004). Indians have poor to moderate vitamin D status and rickets is endemic in Indian children. This may be due to very low vitamin D intakes and dark skin pigmentation (Lips, 2007).

### **2.4.3 Dose-response relationship of serum 25-hydroxyvitamin D to vitamin D intakes**

In order to estimate the intake necessary to achieve a given vitamin D status, knowledge of the relationship between vitamin D intake and circulating 25-hydroxyvitamin

D concentrations is important. A meta-analysis of 16 RCTs that administered vitamin D<sub>3</sub> with or without calcium and measured absolute change in 25(OH)D, demonstrated a significant dose-response relationship between the amount of supplemental vitamin D and serum 25(OH)D concentration [0.016 nmol/L per IU (0.64 nmol/L per µg); *P* = 0.004] (Cranney et al., 2007). This relationship, however is not homogenous (Cranney et al., 2008). As the kinetics of vitamin D are not linear, the dose required to reach a certain serum 25(OH)D concentration depends on pre-existing baseline concentration, subject characteristics (such as age, skin colour, obesity), season and duration of intervention (Cannell et al., 2008). Thus, the exploratory meta-regression analysis controlling for these factors suggested that an increased intake of 100 IU (2.5µg) of vitamin D<sub>3</sub> resulted in an increase of 1–2 nmol/L (0.4– 0.8 ng/ml) in circulating 25(OH)D concentration (Cranney et al., 2008). In addition to the previously mentioned confounding factors, further limitations associated with RCTs assessing the dose-response relationship between 25(OH)D and vitamin D intake include confounding effects from UVB exposure, assay differences and calcium status. For example, the dose-response relationship appears to be dependent on vitamin D status at baseline, with the response to supplementation decreasing with increasing vitamin D status at baseline (Aloia et al., 2008; Viljakainen et al., 2006b). An evidence report covering 26 RCTs and categorising baseline serum 25(OH)D status ( $\leq 40$  vs.  $> 40$  nmol/L) and duration of supplementation ( $\leq 3$  vs.  $> 3$  mo) corroborates this occurrence (Chung et al., 2009). These differences, however, may arise as a result of a regression artifact (regression toward the mean), whereby an initial low baseline 25(OH)D concentration will tend to be closer to the centre of the distribution when measured a second time.

#### 2.4.4 Effect of vitamins D<sub>2</sub> and D<sub>3</sub> on vitamin D status

The two forms of vitamin D, vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, are officially considered to be equivalent, however whether the two forms increase serum 25(OH)D concentrations equally is debated. The suggestion of possible differences with regard to their equivalence was first raised in 1930, when cod liver oil (containing vitamin D<sub>3</sub>) was found to be more effective than Viosterol (vitamin D<sub>2</sub> containing oil) at treating rickets (Hess et al., 1930). However, subsequent studies produced unclear results (Park, 1940), and it was concluded that any effect due to differences was minimal. In 1949 the World Health Organisation recommended that one international unit of vitamin D was equivalent to 0.025 µg (World Health Organisation, 1950), with no distinction to be made between vitamins D<sub>2</sub> and D<sub>3</sub>. As vitamin D<sub>2</sub> and vitamin D<sub>3</sub> have different molecular weights, 396 and 384, respectively, it was recommended in 1972 that one IU should be defined in molecules or moles instead of weight terms. In spite of this, the simple conversion of gram quantity whereby 0.025 µg of either form is equal to one IU is currently in use.

Despite the suggested differences in potencies between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>, both forms have shown to be effective in treating and preventing rickets (via milk fortification) in North America. Thus, if the two have different efficacies, the dosage (per kg body weight) at which vitamin D<sub>2</sub> is given is likely high enough to maintain sufficient bone mineral metabolism (Houghton & Vieth, 2006). With evolution of vitamin D sufficiency evaluation, studies using 25(OH)D concentrations as an objective measure of status, rather than simply comparing the functional outcome or disease endpoint of rickets, have aided in the analytical identification of differences in biological response between the two nutritional forms of vitamin D. These differences have been consistently reported, albeit results have varied (**Table 2.4**). Early studies indicated that vitamin D<sub>3</sub> had greater potency; however

**Table 2.4: Studies comparing the efficacy of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> on improving vitamin D status**

Location	Design	Participants	Assay	Groups	N	Duration	Vitamin D dose	Baseline month/	Baseline 25(OH)D <sup>1</sup> (nmol/L)	Final 25(OH)D <sup>1</sup> (nmol/L)	Change 25(OH)D <sup>1</sup> (nmol/L)
Toronto 43° N (Trang et al., 1998)	Double-blind RCT	Healthy adults	Incstar RIA	D <sub>2</sub> suppl D <sub>3</sub> suppl Placebo	17 55 17	14 days	Equimolar crystalline, in ethanol/ 250nmol (100µg)	Feb-May (winter)	43.7 ± 17.7 41.3 ± 17.7 39.8 ± 18.7	57.4 ± 13.0 <sup>2</sup> 64.6 ± 17.2 <sup>2</sup> 42.8 ± 20.7	13.7 ± 11.4 23.3 ± 15.7 <sup>3</sup> 3.0 ± 8.1
Omaha 41° N (Rapuri et al., 2004)	Cross-sectional	Women 65-77 years	CPBA & HPLC	All D <sub>2</sub> All D <sub>3</sub> No suppl Summer D <sub>2</sub> Summer D <sub>3</sub> Summer no suppl Winter D <sub>2</sub> Winter D <sub>3</sub> Winter, no suppl	56 55 307 21 19 96 32 23 162 no	N/A	401 IU <sup>4</sup> /d 465 IU <sup>4</sup> /d -	Not given	33.0 ± 1.6 <sup>5</sup> 33.6 ± 1.4 <sup>5</sup> 29.3 ± 0.5 32.0 ± 2.1 36.7 ± 2.0 32.2 ± 2.0 33.6 ± 2.3 <sup>5</sup> 29.7 ± 1.8 27.3 ± 0.7		
Nottingham 52° N (Harwood et al., 2004)	RCT	Elderly women after hip fracture	Incstar RIA	IM D <sub>2</sub> IM D <sub>2</sub> + oral Ca Oral D <sub>3</sub> + oral Ca No treatment	38 36 39 37	Final biochem results after 12 mo	300,000 IU 300,000 IU 800 IU/d -	N/A	28 (10-67) 30 (12-85) 29 (6-75) 30 (12-64)	40 <sup>2</sup> 44 <sup>2,3</sup> 50 <sup>2,3</sup> 27	
Omaha 41° N (Armas et al., 2004)	RCT	Healthy adult men	Nichols RIA	D <sub>2</sub> D <sub>3</sub> No treatment	10 10 10	28 days	50,000 IU 50,000 IU -	July	79.2 ± 21.1		-4.6 (-1.7- -10.8) 12 (9.2- 6.7) <sup>3</sup>

Location	Design	Participants	Assay	Groups	N	Duration	Vitamin D dose	Baseline month/	Baseline 25(OH)D <sup>1</sup> (nmol/L)	Final 25(OH)D <sup>1</sup> (nmol/L)	Change 25(OH)D <sup>1</sup> (nmol/L)
Rome 54° N (Romagnoli et al., 2008)	RCT	66-97 yr, female nursing home residents	Diasorin RIA	Oral D <sub>2</sub>	8	30 days	300,000 IU	February	31.5 ± 22.8		43.4 ± 12.0
				IM D <sub>2</sub>	8		300,000 IU		18.3 ± 6.5		12.7 ± 11.2
				Oral D <sub>3</sub>	8		300,000 IU		33.3 ± 24.8		119.5 ± 18.3 <sup>6</sup>
				IM D <sub>3</sub>	8		300,000 IU		20.8 ± 9.0		39.8 ± 28.3
Boston 42° N (Holick et al., 2008)	Double-blind RCT	Healthy adults	LC-MS	D <sub>2</sub>	16	11 weeks	1,000 IU/d	End of winter	42.3 ± 26.3		0.5 ± 13.3
				D <sub>3</sub>	20		1,000 IU/d		49.0 ± 27.8		20.5 ± 19.5 <sup>5</sup>
				D <sub>2</sub> + D <sub>3</sub>	18		500 + 500 IU/d		50.5 ± 26.0		23.3 ± 17.8 <sup>5</sup>
				Placebo	14		-		46.5 ± 22.3		24.8 ± 8.0 <sup>5</sup>
Boston 42° N (Gordon et al., 2008)	RCT	Infants & toddlers with hypovitaminosis D	Diasorin chemiluminescence	D <sub>2</sub> daily	12	6 weeks	2,000 IU/d	October '05 – June '07	39.3 (18-50)		109.8 <sup>2</sup>
				D <sub>2</sub> weekly	14		50,000 IU/wk		34.5 (18-50)		110.0 <sup>2</sup>
				D <sub>3</sub> daily	14		2,000 IU/d		34.3 (18-50)		103.0 <sup>2</sup>
Atlanta 33° N (Khazai et al., 2009)	RCT	Vitamin D insufficient adults with cystic fibrosis	LC-MS	D <sub>2</sub>	10	12 weeks	50,000 IU/wk	Autumn & winter months	61.0 ± 25.8		81.8 ± 24.3
				D <sub>3</sub>	9		50,000 IU/wk		53.0 ± 25.5		117.8 ± 51.3
				UV exposure	9		3-10 mins*5/wk		56.5 ± 27.0		70.8 ± 23.0
London 51° N (Leventis & Kiely, 2009)	RCT	Vitamin D deficient adults	Nichols ELISA	IM D <sub>2</sub>	50	12 weeks	300,000 IU	May	20.3 (<17-35)		32.3 (17-49)
				Oral D <sub>3</sub>	19		300,000 IU		27.0 (5-40) <sup>3</sup>		81.8 (47-119)
											53.8 (23-99) <sup>2,3</sup>

Location	Design	Participants	Assay	Groups	N	Duration	Vitamin D dose	Baseline month/	Baseline 25(OH)D <sup>1</sup> (nmol/L)	Final 25(OH)D <sup>1</sup> (nmol/L)	Change 25(OH)D <sup>1</sup> (nmol/L)
Jos Plateau, Nigeria, 80 ° N (Thacher et al., 2010)	RCT	Rachitic and control children	LC-MS/MS	Rachitic D <sub>2</sub>	16	14 days	50,000 IU	Not given	27.5 (7-16)	72.5 ± 42.5 <sup>7,8</sup>	
				Rachitic D <sub>3</sub>	12		50,000 IU		37.5 (28-60)		
				Control D <sub>2</sub>	11		50,000 IU		65.0 (53-85)		
				Control D <sub>3</sub>	10		50,000 IU		70.0 (38-83)		
Boston 42 ° N (Biancuzzo et al., 2010a; Biancuzzo et al., 2010b)	RCT	Healthy adults	LC-MS/MS	O/ juice D <sub>2</sub>	17	11 weeks	1,000 IU	Mid-Feb	AUC from baseline to 11 wks: 657.3 ± 180.0 <sup>5</sup> 724.3 ± 184.5 <sup>5</sup> 641.5 ± 195.0 <sup>5</sup> 708.0 ± 275.5 <sup>5</sup> 501.3 ± 228.8		
				O/ juice D <sub>3</sub>	18		1,000 IU				
				Oral D <sub>2</sub>	16		1,000 IU				
				Oral D <sub>3</sub>	20		1,000 IU				
				Placebo	15						
Omaha 41 ° N (Heaney et al., 2010)	RCT	Healthy adults	Diasorin chemiluminescence	Oral D <sub>2</sub>	16	12 weeks suppl (18 wk duration)	50,000 IU/wk	Late winter	AUC from baseline to 12 wks: 3400 ± 1290 5340 ± 1640 <sup>3</sup>		
				Oral D <sub>3</sub>	17		50,000 IU/wk				
Madison ° N (Binkley et al., 2011)	RCT	Adults ≥ 65 yrs, comm. dwelling	Reverse phase HPLC	Daily D <sub>2</sub>	16	12 months	1,600 IU	Not given	80.0 ± 5.3	95.3 ± 5.0	
				Daily D <sub>3</sub>	16		1,600 IU		74.8 ± 6.3		
				Monthly D <sub>2</sub>	16		50,000 IU		77.8 ± 5.5		
				Monthly D <sub>3</sub>	16		50,000 IU		90.8 ± 5.3		

<sup>1</sup> mean ± SD OR mean (range)

<sup>2</sup> significantly different from baseline values

<sup>3</sup> significantly different from D<sub>2</sub> supplemented group

<sup>4</sup> average supplemental intakes

<sup>5</sup> significantly different from unsupplemented group

<sup>6</sup> significantly different to other forms of supplements

<sup>7</sup> peak 25(OH)D concentration achieved 3 days after supplementation

<sup>8</sup> significant differences between D<sub>2</sub> and D<sub>3</sub> supplemented groups

these results are inconclusive due to insufficient sample size and the influence of confounding factors (such as seasonal UVB exposure) (Hartwell et al., 1987; Tjellesen et al., 1986; Whyte et al., 1979).

In order to determine differences in biological activity of the two forms of vitamin D, Armas et al., (2004) administered a single oral dose of 50 000 IU (1250 µg) of either vitamin D<sub>2</sub> or vitamin D<sub>3</sub> to healthy adult men (n = 20). Changes in serum 25(OH)D concentrations were measured daily for 28 days and the supplementation groups were compared, following correction for change that occurred in the control population (no vitamin D administered). Over the first three days the increase in 25(OH)D concentrations for the two groups were not significantly different. Serum 25(OH)D levels continued to rise in the vitamin D<sub>3</sub> supplemented group, peaking at day 14 and at day 28 were higher than the peak value for the vitamin D<sub>2</sub>-treated group. In the vitamin D<sub>2</sub> group, 25(OH)D concentration began to decline after day three, reaching baseline values at day 14. A continued decrease was observed with values falling below baseline by day 28. Area under the curve (AUC) was calculated to provide a measure of total exposure, and thus assess the effectiveness of the two supplements. At 28 days the AUC (mean ± SD) for vitamin D<sub>3</sub> was 511.8 ± 80.9 nmol/L, which was significantly greater than the AUC for D<sub>2</sub> (150.5 ± 58.5 nmol/L) (*P* < 0.002) (Armas et al., 2004). The authors proposed that single bolus doses of vitamin D<sub>2</sub> might be three times less effective than equivalent doses of vitamin D<sub>3</sub>; additionally, vitamin D<sub>2</sub> supplementation appears to result in an overall decline in vitamin D status over time.

Recently Romagnoli et al (2008) compared the effect of single dose vitamin D<sub>2</sub> or vitamin D<sub>3</sub> supplements (300 000 IU) on 25(OH)D and PTH concentrations in 32 elderly women over a 60 day period. The two forms of vitamin D were given both intramuscularly or orally at baseline. Oral doses of both vitamin D<sub>2</sub> or vitamin D<sub>3</sub> produced a large

significant increase in 25(OH)D concentration by day three. Increases in 25(OH)D were observed in the intramuscular group beginning at day seven. Continued increases were seen in the oral-vitamin D<sub>3</sub> group and after 30 days, oral vitamin D<sub>3</sub> caused the greatest increase in serum 25(OH)D concentrations and largest decrease in PTH, compared to the other groups ( $P < 0.001$ ). Serum 25(OH)D concentration began to decline in the orally-treated groups after day three, whereas those given intramuscular vitamin D experienced a slow gradual increase throughout the study period. This suggests the intramuscular route may be unable to adequately increase serum 25(OH)D as it is not the physiological route of administration, which needs to be taken into consideration when assessing the efficacy of the two forms of vitamin D. Regardless, both forms of intramuscularly administered vitamin D caused continued increases in 25(OH)D concentrations over the course of the study (Romagnoli et al., 2008). These increases were also greater in the vitamin D<sub>3</sub> treated subjects, suggesting that vitamin D<sub>3</sub> is more potent than vitamin D<sub>2</sub> when given orally or intramuscularly. In contrast to the findings by Armas et al. (2004), vitamin D<sub>2</sub> supplementation did not cause vitamin D status to decline post-supplementation. The effect of vitamin D<sub>3</sub> on reducing PTH levels may be attributed to increased efficacy of this supplemental form as subjects were matched at baseline for 25(OH)D and PTH levels (Romagnoli et al., 2008). The small sample size of the study limits the strength of these findings; however, the results are supported by findings by Leventis and Kiely (2009), who compared the effectiveness of 300 000 IU intramuscular vitamin D<sub>2</sub> (n = 50) to 300 000 IU orally administered vitamin D<sub>3</sub> (n = 19) in treating vitamin D insufficient subjects over winter in the United Kingdom. The mean magnitude of change in serum 25(OH)D concentrations was significantly greater in vitamin D<sub>3</sub>-treated subjects at six and twelve weeks – more than triple the change in vitamin D<sub>2</sub>-treated subjects ( $P < 0.001$ ). The route of administration may account for some of the difference, as 25(OH)D levels declined from 6 to 24 weeks in D<sub>3</sub>-treated subjects, but continued to increase slightly in those given vitamin

D<sub>2</sub> (Leventis & Kiely, 2009). Nonetheless, the magnitude of difference supports the likelihood of high dose vitamin D<sub>3</sub> being more potent. Heaney et al. (2010) also found that 50 000 IU/week oral vitamin D<sub>3</sub>, administered over 12 weeks to healthy adults, was 87% more effective in increasing and maintaining 25(OH)D concentration, compared to an equivalent dose of vitamin D<sub>2</sub> ( $P < 0.001$ ).

Daily supplementation trials have also been undertaken to assess whether there are differences in the efficacy of vitamins D<sub>2</sub> and D<sub>3</sub>. Following two weeks of supplementation with either 4000 IU vitamin D<sub>2</sub> or vitamin D<sub>3</sub>, vitamin D<sub>3</sub> increased 25(OH)D concentrations significantly more than vitamin D<sub>2</sub> (mean difference, 9.6 nmol/L; 95% CI: 1.4, 17.8 nmol/L;  $P = 0.03$ ) (Trang et al., 1998). This study was undertaken during winter, when vitamin D concentrations are low and solar exposure produces minimal vitamin D decreasing the impact of these confounding variables. However, it is unlikely that the two-week study period was long enough to accurately assess the effectiveness of the two forms of vitamin D. Holick et al. (2008) found that 25(OH)D levels began to plateau at six weeks, while other continuous dosing studies have shown that time to equilibrium for vitamin D<sub>3</sub> is three to six months (Heaney et al., 2003a; Mocanu et al., 2009; Patel et al., 2001; Smith et al., 2009). Furthermore, the increase in serum 25(OH)D levels seen in this study was less than expected with a 4000 IU/d dose, which may be due to the unstable ethanol formulation of the supplements (Askew et al., 1932; Holick et al., 2008; Huber & Barlow, 1943).

In order to assess the effect of continued daily supplementation on serum 25(OH)D levels, Holick et al. (2008) supplemented 68 adult subjects with 1000 IU vitamin D/d for 11 weeks; as vitamin D<sub>2</sub>, vitamin D<sub>3</sub> or 500 IU of both at the end of winter. Circulating 25(OH)D levels significantly increased to the same extent in all three treatment groups. They began to plateau by week 6 and after 11 weeks the increases remained very similar in

supplemented groups. The rise in 25(OH)D for all three groups from baseline to the end of the study showed a dose-response relationship whereby serum 25(OH)D concentrations increased by 0.02 nmol/L per IU (0.91 nmol/L per µg) (Holick et al., 2008). These results are consistent with those of Markestad et al. (1984) and Rapuri et al. (2004), who found that vitamin D<sub>2</sub> and vitamin D<sub>3</sub> contributed equally to 25(OH)D concentrations in mothers and their neonates, and elderly women, respectively. Strengths of the study include the use of a placebo group, which demonstrated minimal change in 25(OH)D, suggesting that confounding factors like sun exposure or dietary vitamin D intake had little influence (Holick et al., 2008). Limitations were also noted by authors, as the sample size was underpowered to detect a difference and subjects were permitted to take multivitamin supplements, the majority of which contained vitamin D<sub>3</sub>.

Two smaller studies have compared the effects of combined vitamin D (both D<sub>2</sub> and D<sub>3</sub>) and calcium supplementation on skeletal health outcomes, 25(OH)D and PTH concentrations. In 40 infants and toddlers given 2000 IU vitamin D<sub>3</sub> daily, 2000 IU vitamin D<sub>2</sub> daily or 50,000 IU vitamin D<sub>2</sub> weekly with calcium (50 mg/kg/d) for six weeks, serum 25(OH)D concentrations tripled in all three groups (Gordon et al., 2008). There were no significant differences in serum 25(OH)D, PTH or alkaline phosphatase (bone turnover marker) concentrations between daily and weekly vitamin D<sub>2</sub>-treated groups, or between daily treated vitamin D<sub>2</sub> and D<sub>3</sub> groups. The magnitude of the difference, in combination with the small sample size, negatively affected the power of the study to detect a difference amongst groups.

Harwood et al. (2004) supplemented 87 elderly women with hip fracture with a bolus of 300 000 IU vitamin D<sub>2</sub>, intramuscular vitamin D<sub>2</sub> plus 1000 mg/d oral calcium, or a daily dose of 800 IU oral vitamin D<sub>3</sub> with 1000 mg oral calcium, or placebo. Participants were

followed-up after a one-year period. Twenty percent of patients given intramuscular vitamin D were overtly deficient at the end of the study, and final mean 25(OH)D concentration was 40 nmol/L in this group (Harwood et al., 2004), thus the dose provided may not have been sufficient for the study duration. Despite the potentially low dose, mean 25(OH)D concentrations were significantly higher, PTH was lower and hip BMD was significantly greater in the supplemented groups compared to placebo. The alterations in hip BMD, 25(OH)D and PTH concentrations seen in the orally supplemented vitamin D<sub>3</sub> groups also improved significantly compared to the group that received intramuscular vitamin D<sub>2</sub> alone. The results indicate that vitamin D<sub>3</sub> has greater potency than vitamin D<sub>2</sub>, although the two different forms of supplement administration make it difficult to ratify study conclusions.

In order to compare whether potency of vitamin D<sub>2</sub> and D<sub>3</sub> differs between daily and monthly dosing, Binkley et al. (2011) supplemented 64 elderly ( $\geq 65$  years of age) participants with either 1600 IU daily or 50,000 IU once-monthly of either vitamin D<sub>2</sub> or vitamin D<sub>3</sub> over a 12 month period. Pooled results demonstrated that vitamin D<sub>3</sub> increased serum 25(OH)D by 13% more than vitamin D<sub>2</sub> (95% CI: 3, 23%;  $P < 0.01$ ). Similar increases were seen for both dosing frequencies (daily, 14%; 95% CI: 0, 29%;  $P < 0.05$ ; monthly, 11%; 95% CI: 2, 27%;  $P = 0.11$ ). Furthermore, frequency of dosing did not significantly impact 25(OH)D levels (daily vs. monthly, 5%; 95% CI: -4, 15%;  $P = 0.29$ ). Dosing with vitamin D<sub>2</sub> resulted in increases in 25(OH)D<sub>2</sub> concentration accompanied by a rapid reduction in 25(OH)D<sub>3</sub> of around three-times the corresponding increase in total 25(OH)D concentration. No differences in serum or 24-hour urinary calcium, serum PTH and bone turnover markers were observed in any of the groups. The absence of change in these physiological endpoints means the differences in efficacy of the two forms may not be biologically significant. Nevertheless, this study had some limitations including small sample size ( $n = 16$  in each group), the older age of participants, no indication of when

participants commenced the study (thus there may be a seasonal affect) and that it was not designed to examine differences in PTH concentration, thus the impact on functional outcomes remains unclear.

One of the proposed reasons for the possible differences in serum 25(OH)D concentrations seen in vitamin D<sub>2</sub>- vs. vitamin D<sub>3</sub>-treated subjects is the affinity of DBP for the two forms of vitamin D. The DBP appears to have a greater affinity for 25(OH)D<sub>3</sub> than it does for 25(OH)D<sub>2</sub> (Hollis, 1984; Nilsson et al., 1972), association constants are  $2.8 \times 10^8$  for vitamin D<sub>3</sub> compared to  $1.3 \times 10^8$  for vitamin D<sub>2</sub>(Nilsson et al., 1972). The relative binding of vitamin D and its metabolites for DBP controls their circulating half-lives, consequently if DBP has less affinity for 25(OH)D<sub>2</sub> it would have a shorter circulating half-life than 25(OH)D<sub>3</sub>, and increased clearance from circulation (Houghton & Vieth, 2006). Outcomes from studies where different doses (single vs. daily) are used may be appreciably affected by differences in half-life due to relative binding to DBP. Half-life may be a less important influence in daily dosing studies, as vitamin D is continually administered and thus continues to cause increases 25(OH)D concentrations.

Hepatic 25-hydroxylase may have a higher affinity for vitamin D<sub>3</sub> than vitamin D<sub>2</sub> (Holmberg et al., 1986), which may also account for the potential difference in potency. In the first activation step in the metabolism of vitamin D, mitochondrial vitamin D-25 hydroxylase converts vitamin D<sub>3</sub> to 25(OH)D<sub>3</sub> five times faster than it converts vitamin D<sub>2</sub> to 25(OH)D<sub>2</sub> (Holmberg et al., 1986). The decline in 25(OH)D<sub>3</sub> following dosing with vitamin D<sub>2</sub> supports the theory of substrate competition for the 25-hydroxylase enzyme (Binkley et al., 2011). However, the similar rise in serum 25(OH)D concentrations seen in vitamin D<sub>3</sub> and vitamin D<sub>2</sub> treatment groups over the first three days post-supplementation

suggests that this is less likely to be the reason for the difference in efficacy (Armas et al., 2004; Romagnoli et al., 2008; Thacher et al., 2010).

Following 25-hydroxylation, 25(OH)D and 1,25(OH)<sub>2</sub>D undergo 24-hydroxylation in the kidney and the differences in the side chains of vitamins D<sub>2</sub> and D<sub>3</sub> results in the production of two different metabolites, which accordingly have differing metabolic fates. Vitamin D<sub>2</sub> becomes deactivated when 1,24,25(OH)<sub>3</sub>D<sub>2</sub> is formed, whereas the vitamin D<sub>3</sub> metabolite 1,24,25(OH)<sub>3</sub>D<sub>3</sub> has to undergo an additional hydroxylation to become biologically deactivated (Horst et al., 2005). 1,24,25(OH)<sub>3</sub>D<sub>3</sub> can bind to VDR, potentially generating significant biological activity. 24-hydroxylation of the vitamin D<sub>2</sub> side-chain can also occur in the liver, leading to the synthesis of 24(OH)D<sub>2</sub> (Horst et al., 1990; Mawer et al., 1998), and 1,24(OH)<sub>2</sub>D<sub>2</sub> formed from this also has less affinity for VDR than both 1,25(OH)<sub>2</sub>D<sub>3</sub> and 1,24(OH)<sub>2</sub>D<sub>3</sub> (Horst et al., 2000). Thus, the greater bioefficacy of vitamin D<sub>3</sub> may arise from the higher affinity of its metabolites for VDR, or occur because vitamin D<sub>3</sub> is not directly metabolised to 24(OH)D.

To date, studies undertaken in this area have been limited by confounding effects of seasonal changes in 25(OH)D concentration, short study duration, differences that arise from type (bolus or daily) and route (oral or intramuscular) of administration, small participant numbers, varying vitamin D status at baseline and not controlling for BMI. These limitations have led to conflicting results regarding the efficacy and bioequivalence of vitamin D<sub>2</sub> compared to vitamin D<sub>3</sub>. Large intervention studies that control for confounding factors, have sufficient sample sizes to detect statistical differences and are of adequate duration are required to confirm whether there are differences in potency between the two forms of vitamin D, particularly in the area of daily supplement regimens, are needed.

## **3 Predictors of vitamin D status and its association with parathyroid hormone in healthy New Zealand adults**

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### **3.1 Introduction**

Serum 25-hydroxyvitamin D [25(OH)D] is regarded as the best indicator of vitamin D status. Over the last several years there has been increasing interest in the impact of serum 25(OH)D status on health. The role of vitamin D in bone health is well known (Chung et al., 2009; Cranney et al., 2007; Institute of Medicine, 2010). Emerging epidemiological and experimental evidence also suggests that vitamin D insufficiency may play a role in the pathogenesis of several non-communicable diseases, such as certain cancers, multiple sclerosis and type 1 diabetes (Holick, 2004b; Holick, 2007; Munger et al., 2006; Scientific Advisory Committee on Nutrition, 2007). However, evidence to date is insufficient to establish a serum 25(OH)D cut-off for any non-skeletal disease state. Serum 25(OH)D concentration  $< 30$  nmol/L has a known impact on skeletal health, being associated with calcium malabsorption and secondary hyperparathyroidism, leading to increased bone resorption and osteomalacia in adults and rickets in childhood (Institute of Medicine, 2010; Wasserman, 2005). Fractional calcium absorption appears to be adequate in the range of 30 to 50 nmol/L for most persons, and does not appear to be enhanced further above serum 25(OH)D of 50 nmol/L (Institute of Medicine, 2010). Based on inverse relation between vitamin D status and parathyroid hormone (PTH), optimal vitamin D status has also been defined using the 25(OH)D concentration that maximally suppresses PTH levels. This proposed means of defining vitamin D sufficiency is based on the assumption that the suppression of PTH is favourable for the maintenance of bone mass. Studies to date have

reported a wide range of 25(OH)D concentrations from 25 to 125 nmol/L above which PTH plateaus (Dawson-Hughes et al., 2005; Holick et al., 2005; Vieth et al., 2003), and the optimal concentration of 25(OH)D and PTH for bone health remain unclear (Prentice et al., 2008).

The 1997 Adult Nutrition Survey highlighted concerns that New Zealanders are at risk of suboptimal bone health with nearly 50% of participants having 25(OH)D of less than 50 nmol/L (Rockell et al., 2006). With the exception of oily fish, most foods naturally contain very limited amounts of vitamin D. Moreover, food fortification of vitamin D is neither mandated nor common in New Zealand (Food Standards Australia New Zealand, 2009). Vitamin D acquired through cutaneous synthesis is affected by sunscreen use, clothing/ dress and tanning, and outdoor physical activity (Scientific Advisory Committee on Nutrition, 2007). Season has consistently been identified as a predictor of the vitamin D status of adult New Zealanders, with higher rates of insufficiency observed in winter months compared to summer months (Bolland et al., 2006; Livesey et al., 2007; Lucas et al., 2005; McAuley et al., 1997; Rockell et al., 2005; Rockell et al., 2006). Obesity, age and skin pigmentation are also physiological factors that have an effect on the production of vitamin D following exposure to UV radiation (Bolland et al., 2007; Looker et al., 2002; Snijder et al., 2005).

Several studies have evaluated the vitamin D status of adult New Zealanders (Bartley, 2008; Bolland et al., 2006; Bolland et al., 2007; Chiu, 2005; McAuley et al., 1997; Ogle & Davison, 1985; Rockell et al., 2006), but few have examined the dietary, behavioural and physiological predictors of serum 25(OH)D concentrations. Furthermore, only one study has investigated the relationship of serum 25(OH)D with PTH in this population (Rockell et al., 2008a). Thus, the objectives of the present study were to collect descriptive data and

evaluate the determinants of serum 25(OH)D levels in a group of healthy adults at the end of summer, and to investigate the relationship of serum 25(OH)D and PTH levels.

## 3.2 Participants and Methods

### 3.2.1 Study design and participants

This study used baseline data from a 24-wk double-blinded, randomized controlled trial designed to compare the effect of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> supplements compared to a placebo on serum 25(OH)D concentrations. Ninety-five healthy, adult women and men aged 18–50 y were recruited from the staff and student population at the University of Otago, Dunedin, New Zealand (45°S), and from the community through advertisements in the local newspaper (Appendix A). This region has a temperate climate, with a summer mean temperature of 14°C and a winter mean temperature of 5° and average sunshine hours in the winter ranging from 98–122 hours per month (New Zealand Climate). Participants were excluded if they had a BMI  $\geq$  25, had reported granulomatous conditions, liver disease, kidney disease, or diabetes, were taking medications that might affect vitamin D metabolism (e.g. anticonvulsants, steroids in any form, or barbiturates), or were planning to travel during the course of the study to a location at which the latitude would be predicted to result in cutaneous synthesis of vitamin D.

At the baseline study visit, a non-fasting venous blood sample was collected and participants completed a brief self-administered socio-demographic and health questionnaire (Appendix B). They were asked to report the use of (i) any vitamin D and calcium containing supplements over the past 3 months, (ii) current prescription medications (including oral contraceptives), and (iii) sunless tanning lotion (brand, frequency of use and date last used). Prior to statistical analysis, level of education was divided into two groups: those who had completed a university degree, or those who had done some tertiary study (certificate/diploma, uncompleted university degree) or less. Height was measured to the nearest 0.1 cm using a calibrated self-made stadiometer, and weight was measured to the

nearest 0.1 kg using a calibrated platform digital scale (Seca, Hamburg, Germany), according to standardised procedures (Lohman et al., 1988). Body mass index was determined using the height and weight measurements;

$$BMI = weight(kg)/[height(m)]^2$$

Participants were then given detailed verbal and written instructions on how to collect diet records, and were asked to record all foods and beverages consumed for 5 weekdays and 2 weekend days within the next 14 days. Lastly, participants were asked post-intervention about cigarette smoking and classified according to their current status. Participants were either defined as either current smokers or non-smokers. Non-smokers included those who had never smoked or who had been smoke-free for at least one year and included those who lived in a house where someone else smoked. Ethical approval for the study was obtained from the Human Ethics Committee at University of Otago, Dunedin, New Zealand, and all participants provided written informed consent (Appendix C). The clinical trial was registered at [www.actr.org.au](http://www.actr.org.au) as ACTRN1260-000273280.

### **3.2.2 Dietary Calcium Assessment**

Dietary intake of calcium was determined via a 7-d estimated diet record completed by participants over a 14-d period, including 5 weekdays and 2 weekend days (Appendix D). Seven days of recording is the recommended length required to estimate the average calcium intakes in a group of women (Basiotis et al., 1987). Participants were advised to follow their usual eating patterns, and instructed to record the quantity of each food consumed using household measures and weights provided on packaging. Detailed information regarding brand, preparation and cooking method, and any additions made to the food (such as salt, spices or water) was requested. Dietary calcium intakes were analysed using the software program Diet Cruncher 1999-2001 (version 1.2.0 Ross Marshall-Seeley, Way Down South Software, Dunedin, New Zealand), which incorporates the New Zealand Food Composition

Database (New Zealand Institute of Crop and Food Research, 2004). Dietary calcium intake values were expressed as the average of 7-d of recorded intake.

### **3.2.3 Determination of facultative and constitutive skin colour**

At baseline skin colour was measured by reflectance colourimetry using a Konica Minolta CM-700d spectrophotometer (Konica Minolta Sensing, Inc, Osaka Japan). Constitutive (natural, or genetically inherited at a non-exposed UV site) skin colour was measured on the upper anterior arm and facultative (sun-induced, or tanned) skin colour was taken on the posterior forearm. Prior to taking pigmentation measurements, participants were dry-shaved to ensure the measurement sight was smooth and clear. Measurements were recorded for luminance ( $L^*$ ), the level of gray, from black to white and the yellow-blue component ( $b^*$ ) axes of colour based on the Commission Internationale de l'Eclairage recommended  $L^*a^*b$  system (Weatherall & Coombs, 1992). These data were then used to define individual typology angle ( $ITA^\circ$ ) using the following equation:  $[\arctangent(L^* - 50)/b^*] \times 180/\pi$ . The  $ITA^\circ$  value was used to classify skin colour into one of six groups as follows: very light  $> 55^\circ >$  light  $> 41^\circ >$  intermediate  $> 28^\circ >$  tanned  $> 10^\circ >$  brown  $> -30^\circ >$  dark (Del Bino et al., 2006).

### **3.2.4 Biochemical analyses**

Serum  $25(OH)D_2$  and  $25(OH)D_3$  concentration was determined by isotope-dilution liquid chromatography tandem mass spectroscopy (LC-MS/MS) (Maunsell et al., 2005). Briefly, serum samples (200  $\mu$ L) were denatured with 70% methanol containing stable-isotope-labelled internal standard (IS), vortex-mixed, extracted into hexane, and dried under nitrogen. The reconstituted extract was chromatographed on a Phenomenex (Torrance, CA) C8 reverse phase column [50  $\times$  2.1 mm (i.d.); 5  $\mu$ m particle size], and the metabolites and IS

were detected by electrospray ionization MS/MS in multiple-reaction monitoring mode. The API 3200™ tandem spectrometer and TurboIonSpray™ source were supplied by Applied Biosystems (Carlsbad, CA), and the HPLC system was an Agilent 1100 system comprising a quaternary pump, a vacuum degasser, a temperature-controlled autosampler, and a temperature-controlled column oven. The detection limit for the assay was < 4 nmol/L for both metabolites. Values for serum 25(OH)D reported as less than 4 nmol/L were considered to be zero. To assess accuracy and interassay variability we prepared an internal quality control by adding 25(OH)D<sub>2</sub> and 24(OH)D<sub>3</sub> to a pooled serum sample and ran an external quality control serum material (UTAK Laboratories, Valencia, CA) with a verified 25(OH)D<sub>2</sub> value of 24.2 nmol/L [mean 23.3 (SD 1.2); CV 5.2%] and 25(OH)D<sub>3</sub> value 27.5 nmol/L [mean 25.9 (SD 2.4); CV 9.3%]. For the internal quality control, the interassay CVs for 25(OH)D<sub>2</sub> was 3.8% at 69.8 nmol/L and 4.5% at 86.3 nmol/L. Intact PTH was measured by an automated electrochemiluminescence immunoassay (Elecsys 1010®, Roche Diagnostics, Germany). Serum samples from each subject (for all clinic visits) were analysed together in the same batch. Manufacturer provided controls (Elecsys PreciControl Bone 1, 2 and 3) were analysed with each reagent kit. The mean ± SD (% CV) for the three controls were 45.2 ± 2.8 (6.1%) pg/ml, 155.7 ± 10.3 (6.6%) pg/ml and 650.7 ± 33.5 (5.2%) pg/ml, respectively, and were within the range of the manufacturer provided results. Precision was assessed through the analysis of two or three pooled serum samples in each run (44 serum samples). An interassay CV of 6.2% (n = 29), with a mean (SD) value of 42.2 (2.6) pg/ml was obtained for the pooled plasma.

### **3.2.5 Statistical Analysis**

Statistical analyses were calculated by Stata version 11.0 (Stata Corporation, College Station TX, USA). Baseline socio-demographic characteristics of participants were described using means and standard deviations for continuous variables (i.e. age, weight, BMI), and

percentages for categorical variables. The outcome variables were total serum 25(OH)D and PTH concentration.

To examine factors related to serum 25(OH)D, the following known or potential predictors were identified: age, sex, BMI, level of education, constitutive and facultative skin colour, oral contraceptive use, use of a vitamin D-containing supplement or multivitamin in the three months prior to commencing the study, and current cigarette smoking. Education was collapsed into two categories: secondary school or less or post-secondary education or more. Skin colour was also collapsed into two groups: light (consisting of those with very light and light skin colour as determined by ITA) and tanned (intermediate ITA or darker). Univariate regression models were conducted to examine each of these potential predictors. Variables with  $P < 0.25$  or that had been a priori selected (namely age) were selected for inclusion in the final multivariate regression model to examine the independent contribution of these potential predictors. Statistical significance of the multivariate regression models was determined by  $P < 0.05$ . Serum 25-hydroxyvitamin D concentration was natural log transformed where this resolved issues with skew and/or heteroscedasticity in residuals.

The relationship between serum 25(OH)D and PTH concentrations was evaluated with fractional polynomial regression (Royston & Altman, 1994), controlling for dietary calcium intake. Visual inspection of the resulting relationship was then used to assess whether there was evidence of a plateau in PTH.

**Table 3.1: Baseline characteristics of participants**

	All (n = 95)
Age (y) <sup>1</sup>	28.8 ± 8.6
Range	18 – 50
Females, n (%)	79 (83.2)
Body Mass Index (kg/m <sup>2</sup> ) <sup>1</sup>	22.8 ± 2.5
Ethnicity, n (%)	
NZ European	76 (80.0)
Maori	3 (3.2)
Asian & Indian	5 (5.3)
Other	11 (11.6)
Education, n (%)	
High school	8 (8.4)
Some tertiary	19 (20.0)
Certificate/ diploma	15 (15.8)
University degree	34 (35.8)
Advanced degree	19 (20.0)
Constitutive skin color, n (%)	
Very light	51 (53.7)
Light	36 (37.9)
Intermediate	5 (5.3)
Tanned	3 (3.2)
Facultative skin color, n (%)	
Very light	3 (3.2)
Light	14 (14.7)
Intermediate	38 (40.0)
Tanned	36 (37.9)
Brown	4 (4.2)
Dietary calcium intake (mg) <sup>2</sup>	790.3 (578.1, 1002.0)
Supplemental calcium intake, n (%)	9 (9.5)
Supplemental vitamin D intake, n (%)	8 (8.4)

<sup>1</sup>Arithmetic mean ± SD

<sup>2</sup>Median (1<sup>st</sup>, 3<sup>rd</sup> quartile); Number of participants who completed diet record n = 53

## 3.3 Results

### 3.3.1 Subject demographics

Ninety-five participants were recruited for the study. Baseline socio-demographic, anthropometric and selected dietary characteristics of the study participants are shown in **Table 3.1**. At baseline, the mean age of participants was 29 years (age range 18–50 years) and the majority of participants were New Zealand Europeans (80%), well-educated with at least some tertiary education (92%), and female (83%). Ninety-two percent were classified with very light to light natural (constitutive) skin colour. Facultative (tanned) skin colour, taken at the forearm, was slightly darker with 78% of participants classified as having intermediate or tanned colour skin. All of the participants were classified as normal weight (BMI 18–24.9 kg/m<sup>2</sup>) with a mean (SD) BMI of 23.2 (2.5) kg/m<sup>2</sup>. Median (25<sup>th</sup> and 75<sup>th</sup> percentiles) calcium intake (n = 53 participants completed baseline diet records) was 790 (578, 1002) mg/d and 9 (9.5%) participants regularly took a calcium-containing supplement. Fewer participants (8.4%) reported using a vitamin D containing supplement or multi-vitamin in the three months prior to commencing the study.

Serum 25(OH)D concentrations at baseline ranged from 27.9 to 141.0 nmol/L with the geometric mean serum total 25(OH)D of 74.9 nmol/L. Seven (7.4%) participants had 25(OH)D concentrations below 50 nmol/L, six (6.3%) participants had a serum 25(OH)D concentration ranging between 30–50 nmol/L and one (1.1%) had a serum 25(OH)D < 30 nmol/L.

**Table3.2: Association between selected predictors and serum 25-hydroxyvitamin D concentrations (n=95)**

Explanatory variables	Univariate analysis			Multivariate analysis		
	$\beta^1$	95% CI	<i>P</i> value	$\beta^1$	95% CI	<i>P</i> value
Age	0.07	(-0.43, 0.56)	0.786	-0.02	(-0.52, 0.48)	0.942
Sex <sup>2</sup>	-0.66	(-11.97, 10.65)	0.908			
BMI (kg/m <sup>2</sup> )	-1.04	(-2.76, 0.68)	0.232	-0.71	(-2.48, 1.06)	0.427
Education <sup>3</sup>	2.94	(-5.56, 11.44)	0.494			
Constitutive skin colour <sup>4</sup>	0.05	(-0.39, 0.49)	0.810			
Facultative skin colour <sup>5</sup>	8.60	(-2.30, 19.50)	0.121	9.54	(-1.26, 20.34)	0.083
Use of oral contraceptives	4.68	(-5.03, 14.40)	0.340			
Use of vitamin D supplement	17.43	(2.62, 32.25)	0.022	16.98	(1.26, 32.69)	0.035
Current smoking <sup>6</sup>	0.22	(-20.81, 21.24)	0.984			

<sup>1</sup>Beta coefficient ( $\beta$ ) represents the change in serum 25-hydroxyvitamin D concentration per unit incremental change in the explanatory variable

<sup>2</sup>Females compared to males

<sup>3</sup>Completed university education compared to some tertiary education or less

<sup>4</sup>Very light and light compared to intermediate and tanned

<sup>5</sup>Very light and light compared to intermediate, tanned, brown

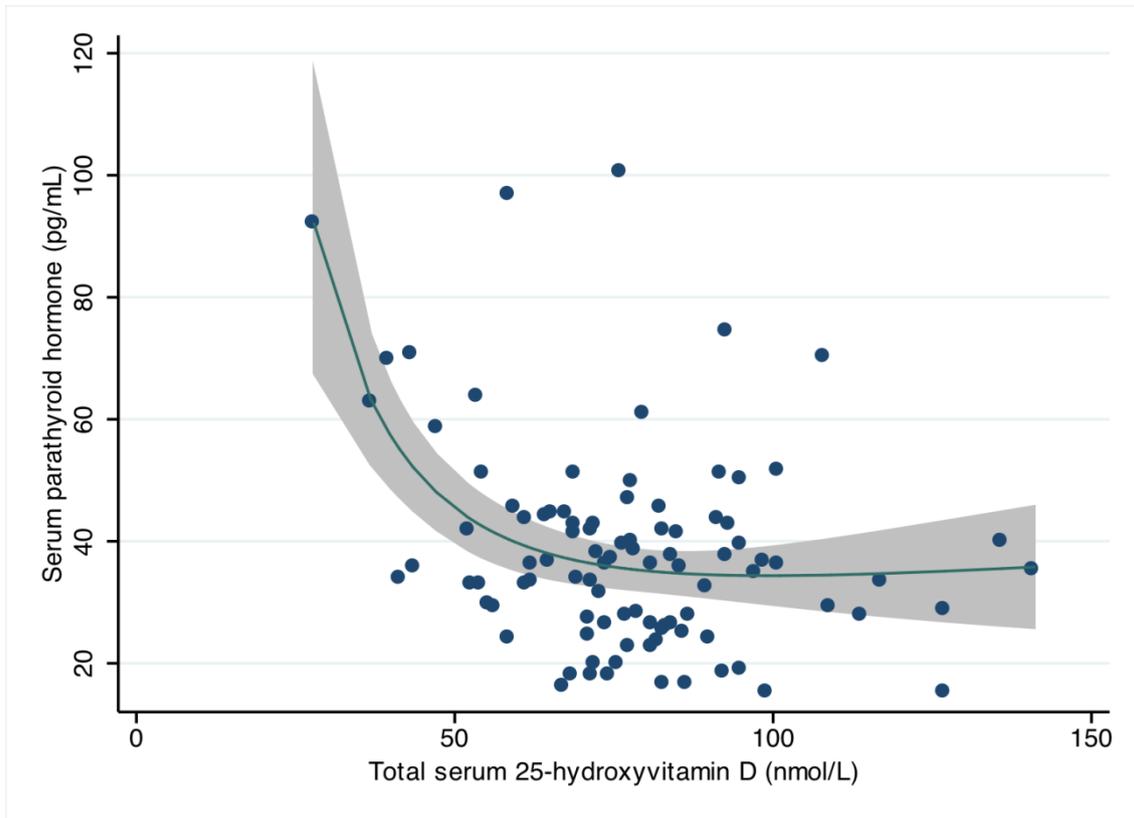
<sup>6</sup>Current smokers compared to non-smokers (including former smokers)

### 3.3.2 Selected predictors associated with serum 25(OH)D concentrations

Regression analyses of the association between predictors and serum 25(OH)D are presented in **Table 3.2**. In the univariate analysis, the use of a vitamin D-containing supplement in the three months prior to commencing the study was positively associated with 25(OH)D concentration ( $P = 0.022$ ). Along with age, BMI and facultative skin colour were also included in the multivariate regression analysis because they appeared to be associated with 25(OH)D concentration ( $P = 0.232$  and  $P = 0.121$ , respectively). In the full multiple linear regression model, only the use of vitamin D supplements prior to commencing the study remained an independent predictor of 25(OH)D concentration ( $P = 0.035$ ). Specifically, mean concentration of 25(OH)D was 17 nmol/L higher in participants who used a vitamin D-containing supplement. None of the following predictors: age, BMI, facultative or constitutive skin colour were associated with serum 25(OH)D concentration in the multivariate model.

### 3.3.3 Association between 25(OH)D and PTH concentrations

Geometric mean (range) serum PTH concentration at baseline was 35.0 pg/ml (15.0, 100.6 pg/ml) ( $n = 95$ ). Eighty-five (89.5%) participants had serum PTH within the normal range (10–60 pg/ml), and seven (7.4%) participants had serum PTH > 65 pg/ml (secondary hyperparathyroidism). Serum PTH concentrations declined in a non-linear manner as serum 25(OH)D concentrations increased. Fractional polynomial regression controlling for dietary calcium intake showed that there was a statistically significant association between serum 25(OH)D and PTH concentrations with 5.4% variation explained by the model ( $P < 0.001$ ). Visual inspection suggests that an increase in serum PTH occurs at a serum 25(OH)D concentration of approximately less than 70 nmol/L (**Figure 3.1**). With the use of this threshold, 31 (32.6%) participants had serum 25(OH)D concentrations < 70 nmol/L.



**Figure 3.1:** Plot of the relationship between total serum 25-hydroxyvitamin D and parathyroid hormone concentrations, with model-estimated line fitted to the data using fractional polynomial regression, controlling for dietary calcium intake.

### **3.4 Discussion**

In this study of healthy adults performed at the end of the summer months in Dunedin, New Zealand (latitude 45°S), the mean 25(OH)D concentration of 75 nmol/L of the sample group reflected adequate status for most, and was slightly higher compared to the average 25(OH)D summer (Dec–Feb) concentrations of 67 and 70 nmol/L, respectively for females and males reported in the 1997 New Zealand Adult Nutrition Survey (Rockell et al., 2006). The only determinant of serum 25(OH)D status during this seasonal period was supplemental intake of vitamin D. Participant characteristics known to influence 25(OH)D status such as BMI, age and skin pigmentation were not evident in this sample group. The threshold concentration for 25(OH)D at which PTH concentration was maximally suppressed appears to be approximately 70 nmol/L.

#### **3.4.1 Predictors of vitamin D status**

Both latitude and season are known determinants of vitamin D status, however in this study all participants were recruited in March (early autumn), and thus, the only significant predictor was the use of a vitamin D containing supplement in the three months prior to study commencement. The association between 25(OH)D concentration and vitamin D supplement use is consistent with other studies (Brock et al., 2010; Burgaz et al., 2007; Gozdzik et al., 2008; Van Dam et al., 2007). Dietary vitamin D intake has also been found to be associated with 25(OH)D concentration (Abbas et al., 2008b; Barake et al., 2010; Chapuy et al., 1997; Holvik et al., 2005), although dietary intake in New Zealand is likely to be low as there is no mandatory fortification and limited voluntary fortification of foods with vitamin D (Food Standards Australia New Zealand, 2009).

The lack of effect of either constitutive (natural) or facultative (tanned) skin colour on 25(OH)D concentration is somewhat unexpected. Melanin competes with 7-dehydrocholesterol for ultraviolet (UV) photons, essentially acting as a UV filter (Chen et al., 2007; Matsuoka et al., 1991). Darker skinned individuals produce less cholecalciferol in the skin for the same exposure to UVB light than lighter-skinned individuals. As a result, they require longer sun exposure to achieve the same serum 25(OH)D concentration. It is therefore expected that those with darker constitutive skin colour would have lower 25(OH)D concentrations (Ginde et al., 2009; Langlois et al., 2010). In a group of healthy US adults, Armas et al. (2007) found that lighter constitutive skin colour was associated with a higher 25(OH)D concentration at baseline ( $r = 0.431$ ,  $p < 0.01$ ) and that less UVB exposure was required to increase 25(OH)D by 30 nmol/L [ $39 \text{ mJ/cm}^2$  3 times a week, for 4 weeks, in those with an  $L^*$  value of 70 (European) vs.  $55 \text{ mJ/cm}^2$  in those with an  $L^*$  score of 50 (African American)]. Conversely, Rockell et al (2008b) found no association between constitutive skin colour and 25(OH)D concentrations in Pacific and European New Zealanders, which the authors suggested may be due to the small number of participants with 'tanned', 'brown' or 'dark' constitutive skin colour. This is also a limitation present in our study, as 92% of participants had 'very light' or 'light' skin colour. The behavioural habits of those with fair skin may also partly explain why facultative skin colour has not reliably been found to predict higher 25(OH)D concentration. Those with fairer skin (phototypes) have been found to have worse vitamin D status than those with darker skin, which may be because they avoid the sun due to greater risk of sunburn (Malvy et al., 2000). They are also more likely to use sunscreen in summer months (Macdonald et al., 2011).

Alternatively, the association of facultative skin colour and serum 25(OH)D has been investigated as darker facultative skin colour is presumed to reflect greater UV exposure, suggesting higher vitamin D status. Tangpricha and colleagues (2004) found that those who

regularly use tanning beds ( $n = 50$ ), and thus have darker facultative skin colour, had higher serum 25(OH)D concentrations compared to non-tanners ( $n = 106$ ). Forehand skin reflectance (measure of facultative skin colour) was found to be a better predictor of 25(OH)D than inner arm reflectance (constitutive skin colour), and explained 30% ( $P < 0.001$ ) of the variation in 25(OH)D concentration in 72 healthy adults of varied ancestry (Hall et al., 2010). Furthermore, in this study UVB dose (sun exposure) was also a predictor of 25(OH)D concentration ( $P < 0.001$ ), although there was no significant interaction between these predictors. Rockell et al. (2008b) also confirmed that darker facultative skin colour was associated with higher serum 25(OH)D concentrations such that 10° lower ITA was associated with a 5 nmol/L higher 25(OH)D concentration ( $P < 0.001$ ). In Caucasian women, change in skin colour from summer to winter was also found to be a major predictor of seasonal change in 25(OH)D concentration, accounting for 13.2% of the total variation in 25(OH)D (Macdonald et al., 2011).

A further limitation seen in our study may be the relatively high 25(OH)D concentrations in this population, as baseline measurements were taken at summertime peak. Constitutive skin colour (melanin concentration) may not be a limiting factor in summer when UV light is plentiful (Rockell et al., 2008b). Thus the limitations of high 25(OH)D concentrations and comparatively few participants with dark skin colour may eclipse the relationship between vitamin D status and skin colour, which already has several other behavioural factors that may also influence the association.

Neither age nor BMI were found to be predictors of vitamin D status in this population. Previous studies have shown that obese individuals ( $BMI > 30 \text{ kg/m}^2$ ) have lower plasma concentrations of 25(OH)D compared to those of a normal weight ( $BMI < 25 \text{ kg/m}^2$ ) (Brock et al., 2010; Hey et al., 1982; Liel et al., 1988). Furthermore, percentage body

fat is inversely associated with serum 25(OH)D concentration in healthy women (Arunabh et al., 2003). This finding may be due to decreased bioavailability of vitamin D caused by increased sequestration in body fat tissue (Looker, 2007; Wortsman et al., 2000). Our study population, however, had a narrow BMI range [mean (SD) 22.8 (2.5)], as normal weight individuals were specifically recruited. The decline in 25(OH)D associated with BMI may therefore only be notable in those with high BMIs who have substantially greater percentage body fat, rather than being seen across all BMI ranges. Other studies support this finding whereby BMI has not been found to be a predictor of vitamin D status in normal weight participants (Gozdzik et al., 2008; Hall et al., 2010). The lack of association between age and vitamin D status was not surprising as participants aged  $\leq 50$  years were recruited. It has been suggested that the prevalence of vitamin D deficiency is high in the elderly due to a decrease in skin 7-dehydrocholesterol concentration (Atli et al., 2005; MacLaughlin & Holick, 1985; Papapetrou et al., 2007). The mean (SD) age of participants in this study was 28.8 (8.6) years, thus it is unlikely that 7-dehydrocholesterol concentrations had begun to decline. Research investigating the dose response relation between vitamin D intake and serum 25(OH)D levels has found that the 25(OH)D status does not appear to vary with age in those aged less than 80 years (Cashman et al., 2008; Cashman et al., 2009; Harris & Dawson-Hughes, 2002).

### **3.4.2 Association between 25(OH)D and PTH concentrations**

Our data suggest that a serum 25(OH)D concentration of around 70 nmol/L is needed to maximally suppress PTH concentrations in calcium sufficient healthy adults. This observation is consistent with other studies undertaken in populations with similar dietary calcium intakes, where the 25(OH)D threshold concentration was between 70 and 80 nmol/L (Aloia et al., 2006; Chapuy et al., 1997; Dawson-Hughes et al., 2005; Holick, 2007). However, other studies have reported threshold estimates between 40 and 50 nmol/L (Aloia

et al., 2005; Durazo-Arvizu et al., 2010; Harris et al., 2000; Need et al., 2004; Need et al., 2000; Steingrimsdottir et al., 2005). Differences in threshold estimates may be due to several factors, including differences in calcium intake, health status, ethnicity and age of the populations studied and variation in methods used to determine 25(OH)D concentration. Aloia and colleagues (2006) conducted a systematic review of studies undertaken to evaluate the relationship between 25(OH)D and PTH and concluded that the threshold level for serum 25(OH)D varied depending on dietary calcium intakes [and vitamin D status (Aloia et al., 2006)]. Among the 18 available publications with dietary calcium information representing a collective sample of 7176 participants, multiple regression analyses found that serum 25(OH)D and dietary calcium intake independently influenced the reported threshold, accounting for around 67% of the variance among the reported thresholds. More recent studies have also confirmed that the 25(OH)D–PTH association is affected by calcium intake (Aloia et al., 2010; Thomas et al., 2010). Furthermore, Aloia and colleagues (2006) found that there was a linear association between mean serum 25(OH)D concentration and PTH threshold, with an almost one-to-one increase in the computed threshold for each 1-nmol/L increase in the mean 25(OH)D concentration. The interrelationship between vitamin D, calcium and PTH, essential for maintenance of serum calcium and bone mineralisation, may explain why the PTH threshold level differs depending on calcium intake and vitamin D status as PTH may be decreased by increasing calcium intake or by increasing vitamin D. Thus, the relatively high baseline serum 25(OH)D concentration of our participants may have led to a higher threshold estimate of the 25(OH)D level at which PTH concentration plateaus.

The mathematical approach most commonly used to determine the relation between 25(OH)D and PTH has been questioned as it presumes a plateau exists, and aims to determine the concentration at which it occurs. As this is not an objective method of

modelling the relationship Vieth and El-Hajj Fuleihan (2005) have questioned whether such a plateau exists. Two recent studies have used a variety of statistical models to determine whether or not a threshold level of PTH is reached (Aloia et al., 2006; Durazo-Arvizu et al., 2010). To help reduce a priori assumptions about the shape of the curve that describes the relationship between PTH and 25(OH)D, Aloia et al. (2006) used three different statistical regression models: Loess, Spline and Exponential decay, and found that a consistent threshold level of 49–50 nmol/L 25(OH)D was reached with each of the models. Durazo-Arvizu and colleagues (2010) also attempted to remove bias around the statistical assumption of a plateau by using a three-phase regression model to estimate the threshold of 25(OH)D and PTH. Using the three-phase model, two thresholds of 30 and 70 nmol/L were found, which relate to the rate of decline in PTH concentration. Prior to the lower threshold, PTH concentrations rapidly decline at a constant rate with increasing 25(OH)D concentration. Once this threshold is reached, PTH concentrations decline at a slower rate until a second (higher) threshold is reached, after which PTH is maximally suppressed. These studies support the statistical reality of a threshold point, regardless of the level of 25(OH)D at which it occurs.

Although we acknowledge that the form of the mathematical relation between PTH and 25(OH)D may influence the threshold point, our study has several strengths including accounting for dietary calcium intakes, investigating the relationship in a sample of healthy, non-elderly adults, and the use of a higher-order reference method (LC-MS/MS) to determine 25(OH)D concentration. Nonetheless, the clinical utility of determining a threshold level indicative of optimal 25(OH)D concentration is not supported by studies assessing bone resorption markers and bone mineral density (Molgaard et al., 2010; Viljakainen et al., 2006a). Specifically, no correlation has been found between serum 25(OH)D and rates of bone loss (Gannage-Yared et al., 2000). Additionally, no significant

differences in bone mineral density were seen in participants with serum 25(OH)D levels above and below a threshold level of 40–50 nmol/L (Aloia et al., 2006).

Despite a lack of support for this threshold level being indicative of optimal vitamin D status for bone health, the role of optimal vitamin D status in non-calcaemic health outcomes is still important to consider. Further research, including clinical trials, needs to focus on the role of vitamin D status and affect of improving status on the prevention of non-skeletal diseases, such as cancers, type 1 diabetes mellitus and heart disease.

In conclusion, supplemental vitamin D intake was associated with vitamin D status, even in periods where there is ample UVB opportunity. Thus, in countries like New Zealand where there is little vitamin D obtained from food sources, supplements may be a valuable source of vitamin D and help improve the vitamin D status of the population. Further work needs to be undertaken in populations with similar behavioural habits and sufficient variation in skin colour to clarify the associations between constitutive and facultative skin colour and 25(OH)D concentration. There also appears to be an inverse relationship between PTH and 25(OH)D levels, whereby a rise in PTH appears to occur below a 25(OH)D concentration of 70 nmol/L. The implications of this relationship for bone health warrant additional research.

## **4 Vitamin D<sub>3</sub> is more effective than vitamin D<sub>2</sub> in maintaining vitamin D status over the winter months**

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### **4.1 Introduction**

During recent years, a number of studies have been performed in humans investigating the relative potencies of the two commonly used forms of vitamin D, ergocalciferol (vitamin D<sub>2</sub>) and cholecalciferol (vitamin D<sub>3</sub>) (Armas et al., 2004; Heaney et al., 2010; Holick et al., 2008; Rapuri et al., 2004; Romagnoli et al., 2008; Tjellesen et al., 1986; Trang et al., 1998). Vitamin D<sub>3</sub>, the form produced in the skin of humans after exposure of 7-dehydrocholesterol to sunlight, is found either naturally in animal products such as fatty fish and cod liver oil, or added as a fortificant to foods. Commercial production of vitamin D<sub>3</sub> is performed by UV irradiating 7-dehydrocholesterol extracted from the lanolin of sheep wool. Vitamin D<sub>2</sub> is primarily human-made from the UV irradiation of ergosterol obtained from yeast, and added to foods. Structurally, vitamin D<sub>2</sub> differs from vitamin D<sub>3</sub> in that its side chain has an added methyl group on carbon 24 and additional double bond between carbons 22 and 23. This difference, however, does not preclude the metabolic activation of the two forms. Prior to exerting their biological effects, both vitamin D<sub>2</sub> and vitamin D<sub>3</sub> must undergo 25-hydroxylation in the liver to form 25-hydroxyvitamin D [25(OH)D; D designates D<sub>2</sub> or D<sub>3</sub>] followed by 1 $\alpha$ -hydroxylation to produce the respective biologically active metabolites 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D].

With the use of serum 25(OH)D as an indicator of vitamin D status and appropriate assay systems to detect 25(OH)D<sub>2</sub> metabolite, several studies have suggested that vitamin D<sub>2</sub> is less effective in elevating or maintaining total serum 25(OH)D levels in healthy adults when administered at doses ranging from 1000 IU to 50 000 IU (Armas et al., 2004; Binkley et al., 2011; Heaney et al., 2010; Rapuri et al., 2004; Romagnoli et al., 2008; Tjellesen et al., 1986; Trang et al., 1998). Holick et al. (2008), however, reported that a daily dose of 1000 IU vitamin D<sub>2</sub> was as effective as 1000 IU vitamin D<sub>3</sub> in maintaining serum 25(OH)D levels suggesting that at lower doses there is no differences in the effects of vitamin D<sub>2</sub> and vitamin D<sub>3</sub>. In addition to varying dose and dosing regimens, studies to date have been limited by short study duration; small participant numbers; and the confounding effects of cutaneous production of vitamin D<sub>3</sub>, background dietary and supplemental vitamin D intake and body mass index.

In an attempt to minimize the effects of confounding variables, we designed a study to evaluate the relative potencies of a daily physiological dose of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> to healthy weight adults for a 6 mo period beginning at the end of summer. For this purpose we specifically investigated the time course of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> serum levels and the concomitant variations in parathyroid hormone concentration after initiation of vitamin D supplementation. The study was conducted in New Zealand, at a latitude of 45°S, where food fortification of vitamin D is neither mandated nor common.

## 4.2 Participants and Methods

### 4.2.1 Study design and participants

The study design, location and participants have been described in **Section 3.2.1**. In brief, the study was a 24 wk double-blinded, randomized controlled trial conducted in Dunedin, New Zealand (45°S), over a 24 wk period, between March 2009 (early autumn) and September 2009 (late winter). Ninety-five healthy, adult women and men aged 18–50 y were recruited from the community, and exclusion criteria included factors that may influence vitamin D status, as outlined previously.

Socio-demographic, health and anthropometric data was collected at baseline (Appendix B), along with a non-fasting venous blood sample, as described in **Section 3.2.4**. Participants were also asked to complete a seven day diet record (Appendix D). Participants were then randomly assigned to receive one of three daily tablets labelled to contain 1000 IU (25 µg) vitamin D<sub>2</sub>, 1000 IU (25 µg) vitamin D<sub>3</sub> or a placebo. Randomisation of participants was completed with a computer-generated block randomisation scheme stratified by sex with a block size of 5. All the participants began the study between 6 March and 13 March 2009 (early autumn) and completed the study 24 wk later between 27 August and 14 September 2009 (late winter).

Participants provided further non-fasting blood samples at clinic visits in wk 4, 8, 12 and 24, and updated information regarding prescription medication and travel was also collected (Appendix C). The study was approved by the Human Ethics Committee at University of Otago, and all participants provided written informed consent (Appendix C).

#### **4.2.2 Study Supplements**

The vitamin D and placebo supplements were manufactured as hard tablets by New Zealand Nutritionals Ltd (Christchurch, New Zealand), and were identical in appearance. Participants were instructed to take one tablet per day. The vitamin D content of the tablets was independently verified on 19 August 2009 (New Zealand Laboratory Services Ltd, Auckland, NZ). Ten tablets were chosen at random from each group and sent to Auckland for analysis. The actual amounts of vitamin D in the tablets labelled to provide 1000 IU (25 µg) of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> were 1295 IU (32 µg) and 1110 IU (28 µg), respectively. The placebo tablets contained no vitamin D. Participants were provided with a sufficient supply of their randomly assigned tablets, and instructed to return all unused tablets. Compliance was assessed using cumulative pill counts at the end of the study. The allocation of participants to arms of the study remained concealed from all researchers until the final analyses were completed.

#### **4.2.3 Dietary Calcium Assessment**

The method for estimating dietary calcium intake was also outlined in **Section 3.2.2**. Briefly, participants completed a 7-d estimated diet record, completed over a 14-d period. The software program Diet Cruncher 1999-2001, version 1.2.0 (Marshall-Seeley, 1999-2001) along with the New Zealand Food Composition Database (New Zealand Institute of Crop and Food Research, 2004) was then used to evaluate dietary calcium intakes.

#### **4.2.4 Determination of facultative and constitutive skin colour**

Reflectance colourimetry was used to measure skin colour at baseline (Konica Minolta CM-700d spectrophotometer, Konica Minolta Sensing, Inc, Osaka Japan), as

previously described in **Section 3.2.3**. The Commission International de l'Eclairage L\*a\*b system (Weatherall & Coombs, 1992) for measuring axes of colour was used to define ITA° values. Skin was then organized into the following groups based on ITA°: very light > 55° > light > 41° > intermediate > 28° > tanned > 10° > brown > -30° > dark (Del Bino et al., 2006).

#### **4.2.5 Biochemical analyses**

As explained in **Section 3.2.4**, serum 25(OH)D concentration was determined by isotope-dilution liquid chromatography tandem mass spectroscopy (LC-MS/MS) (Maunsell et al., 2005). Reconstituted serum samples were chromatographed on a Phenomenex (Torrance, CA) C8 reverse phase column [50 × 2.1 mm (i.d.); 5 µm particle size], and the metabolites and IS were detected by electrospray ionization MS/MS in multiple-reaction monitoring mode. The API 3200™ tandem spectrometer and TurboIonSpray™ source were supplied by Applied Biosystems (Carlsbad, CA), and the HPLC system was an Agilent 1100 system. The detection limit for the assay was < 4 nmol/L and values for serum 25(OH)D reported as less than this were considered to be zero. Mean interassay % coefficients of variation (CV) for external quality control serum 25(OH)D (UTAK Laboratories, Valencia, CA) was 7.3% and for the internal quality control sample the average interassay CV for 25(OH)D was 4.2%.

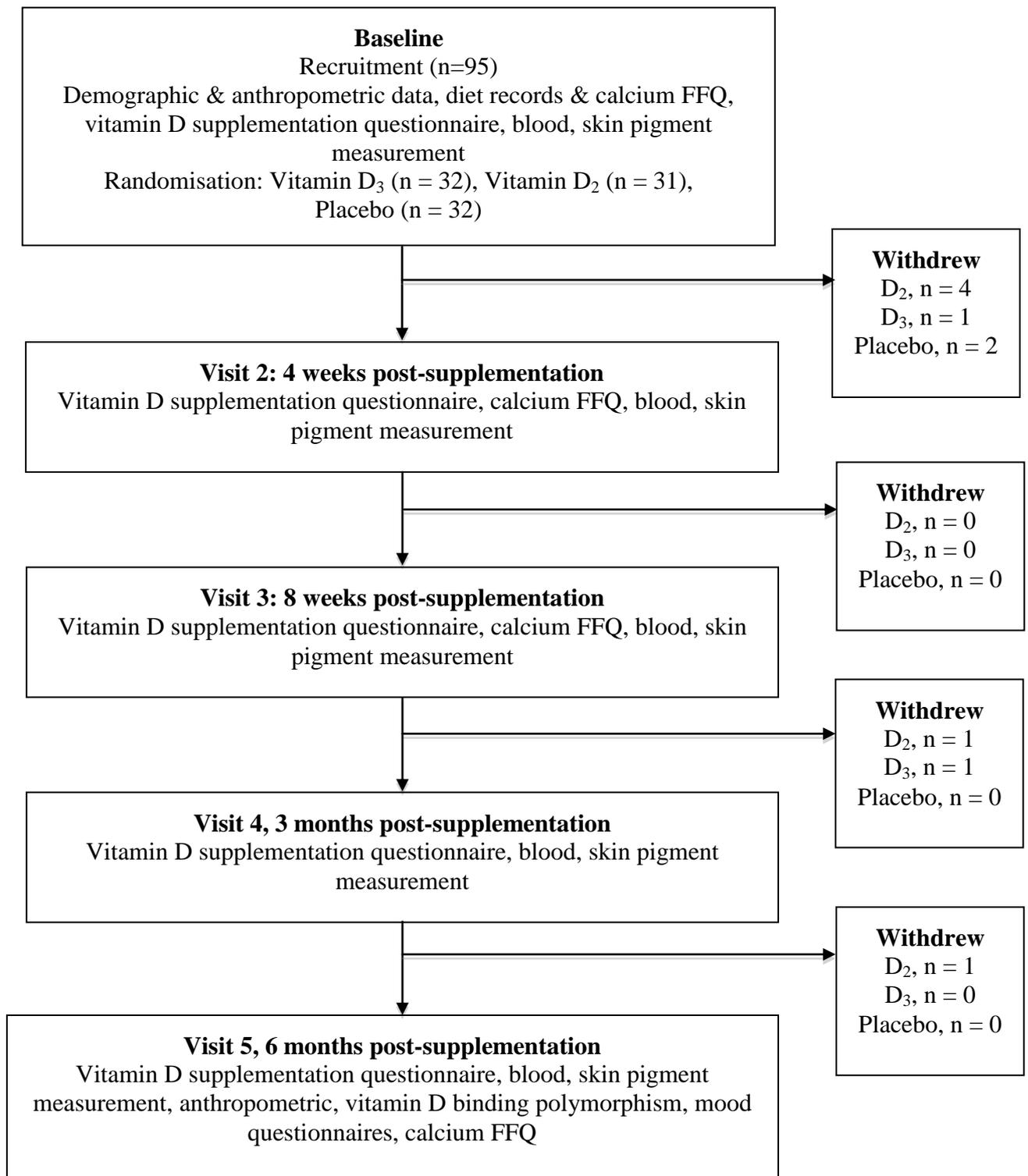
An automated electrochemiluminescence immunoassay (Elecsys 1010®, Roche Diagnostics, Germany) was used to determine serum PTH concentration. Manufacturer provided controls (Elecsys PreciControl Bone 1, 2 and 3) were analysed with each reagent kit and average CV for the three controls was 6.0%, and all three were within the range of the manufacturer provided results.

#### 4.2.6 Statistical Analysis

The sample size for this study was based on a previous study of 25(OH)D concentration in a population of healthy adults (Heaney et al., 2003a). In order to have 90% power to detect a difference of 10 nmol/L (approximately equal to the winter decline) in follow-up serum 25(OH)D concentrations between any pair of treatment arms using a two-sided test at the 0.05 levels, and assuming a standard deviation of approximately 11 nmol/L, 26 participants be required in each arm without making any assumptions about the correlations between baseline and follow-up measurements. Allowing for loss to attrition of 10%, 30 participants would be required in each at baseline.

The outcome variables were total serum 25(OH)D, 25(OH)D<sub>3</sub> and parathyroid hormone concentration. Mixed-effects linear regression models were used to evaluate the fixed effect of the intervention on total serum 25(OH)D, serum 25(OH)D<sub>3</sub> and PTH, including a random subject effect to account for the repeated measures within subjects and controlled for baseline levels. Models with PTH as an outcome also included dietary calcium. Natural log transformations were used where this improved residual normality and/or homoscedasticity. Difference in changes between groups was assessed using combinations of treatment group effects and group-by-time interactions. Selected error covariance structures were investigated and retained where this improved the Akaike information criterion (AIC) for the model under investigation, namely autoregressive, exponential, unstructured, banded, and toeplitz structures. In addition to the intention to treat analysis, a per protocol analysis was undertaken to account for potential differences in compliance amongst treatment groups. A cut-off of 90% compliance was used. The same mixed-effects linear regression models were used in this per protocol analysis.

Non-linear regression analysis was used to estimate the intent-to-treat response of serum 25(OH)D<sub>2</sub> over time in the vitamin D<sub>2</sub> arm of the study. This regression model included vitamin D<sub>2</sub> intake as the only independent variable. The proportion of 25(OH)D<sub>2</sub> in serum as a function of time was modelled with the equation:  $C(t) = C(\infty) - [C(\infty) - C(0)] e^{-kt}$  where  $C(t)$  is the serum 25(OH)D<sub>2</sub> after  $t$  days,  $C(\infty)$  is the final steady state proportion,  $C(0)$  is the initial proportion and  $k$  is the rate constant. Bootstrapping was used to obtain CIs for estimated time required to reach 90% of the estimated plateau on the basis of 5000 bootstrapped samples with a 95% CI obtained using the percentile method. All analyses were based on intention to treat and so compliance is reported by not used in modelling. Stata (version 11.0 Stata Corporation, College Station TX, USA) was used for all analyses and two-sided  $P < 0.05$  was considered statistically significant in all cases.



**Figure 4.1:** Flow diagram of participants through the study

### 4.3 Results

Ninety-five participants were recruited for the study, and 85 participants completed the intervention trial (1000 IU vitamin D<sub>2</sub>/d, n = 25 out of 31 enrolled; 1000 IU vitamin D<sub>3</sub>/d, n = 30 out of 32; placebo, n = 30 out of 32) (**Figure 4.1**). Reasons for withdrawal were as follows: lack of time (n = 3), personal reasons (n = 1), unspecified health reasons (n = 4); and exacerbation of eczema (n = 2). Baseline socio-demographic, anthropometric, dietary and biochemical characteristics of the study participants are shown in **Table 4.1**. Briefly, 95 participants were recruited, the mean age was 29 years and most were New Zealand Europeans (80%) and well educated. Ninety-two percent were classified with very light to light natural (constitutive) skin colour. Facultative (tanned) skin colour, taken at the forearm, was slightly darker with 78% of participants classified as having intermediate or tanned colour skin. All of the participants were classified as normal weight (BMI 18–24.9 kg/m<sup>2</sup>) with a mean (SD) BMI of 23.2 (2.5) kg/m<sup>2</sup>. Median (25<sup>th</sup> and 75<sup>th</sup> percentiles) calcium intake was 790 (578, 1002) mg/d and 9.5% of participants regularly took a calcium-containing supplement. Compliance was high in participants who completed the study with mean (SD; range) compliance of 93% (9%; 63–100%).

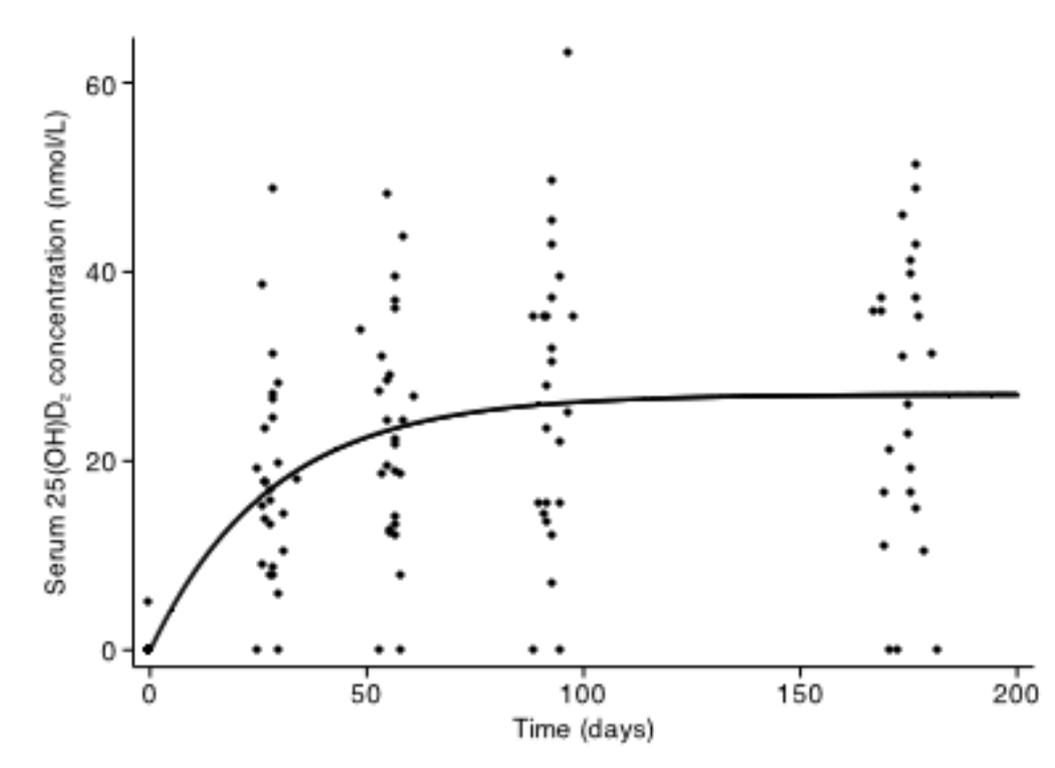
Serum 25(OH)D concentrations at baseline ranged from 27.9 to 141.0 nmol/L with the geometric mean serum total 25(OH)D of 74.9nmol/L. Seven (7.4%) participants had 25(OH)D concentrations below 50 nmol/L. Secondary hyperparathyroidism, defined as a PTH concentration > 65 ng/L (65 pg/mL) was observed in 7 of 95 (7.0%) of the participants at baseline. The concentration of 25(OH)D<sub>2</sub> at baseline was below detectable limits (< 4 nmol/L) for most participants, thus the contribution of 25(OH)D<sub>2</sub> to total 25(OH)D

**Table 4.1: Baseline characteristics of participants**

	All (n = 95)	Treatment group		
		Vitamin D <sub>3</sub> (n = 32)	Vitamin D <sub>2</sub> (n = 31)	Placebo (n = 32)
Age (y) <sup>1</sup>	28.8 ± 8.6	31.0 ± 9.9	26.8 ± 8.0	28.6 ± 7.6
Range	18 – 50	19 – 50	18 – 47	18 – 46
Females, n (%)	79 (83.2)	26 (81.3)	27 (87.1)	26 (81.3)
Body Mass Index (kg/m <sup>2</sup> ) <sup>1</sup>	22.8 ± 2.5	23.2 ± 2.5	22.5 ± 2.5	22.6 ± 2.5
Ethnicity, n (%)				
NZ European	76 (80.0)	27 (84.4)	22 (71.0)	27 (84.4)
Maori	3 (3.2)	1 (3.1)	0 (0.0)	2 (6.3)
Asian & Indian	5 (5.3)	0 (0.0)	5 (16.2)	0 (0.0)
Other	11 (11.6)	4 (12.5)	4 (12.9)	3 (9.4)
Education, n (%)				
Highschool	8 (8.4)	4 (12.5)	2 (6.5)	2 (6.3)
Some tertiary	19 (20.0)	7 (21.9)	9 (29.0)	3 (9.4)
Certificate/ diploma	15 (15.8)	5 (15.6)	3 (9.7)	7 (21.9)
University degree	34 (35.8)	8 (25.0)	12 (38.7)	14 (43.8)
Advanced degree	19 (20.0)	8 (25.0)	5 (16.1)	6 (18.8)
Constitutive skin color, n (%)				
Very light	51 (53.7)	20 (62.5)	10 (32.3)	21 (65.6)
Light	36 (37.9)	10 (31.3)	17 (54.8)	9 (28.1)
Intermediate	5 (5.3)	1 (3.1)	2 (6.5)	2 (6.3)
Tanned	3 (3.2)	1 (3.1)	2 (6.5)	0 (0.0)
Facultative skin color, n (%)				
Very light	3 (3.2)	2 (6.3)	1 (3.2)	0 (0.0)
Light	14 (14.7)	3 (9.4)	4 (12.9)	7 (21.9)
Intermediate	38 (40.0)	13 (40.6)	13 (41.9)	12 (37.5)
Tanned	36 (37.9)	13 (40.6)	11 (35.5)	12 (37.5)
Brown	4 (4.2)	1 (3.1)	2 (6.5)	1 (3.1)
Dietary calcium intake (mg) <sup>2</sup>	790.3 (578.1, 1002.0)	801.4 (733.2, 1002.0)	737.3 (503.6, 1065.2)	772.4 (634.6, 983.7)
Supplemental calcium intake, n (%)	9 (9.5)	4 (12.5)	2 (6.5)	3 (9.4)
Supplemental vitamin D intake, n (%)	8 (8.4)	1 (3.1)	3 (9.7)	4 (12.5)

<sup>1</sup>Arithmetic mean ± SD<sup>2</sup>Median (1<sup>st</sup>, 3<sup>rd</sup> quartile); Number of participants who completed diet record: All, n=53; vitamin D<sub>3</sub>, n=18; vitamin D<sub>2</sub>, n=17; placebo, n=16.

concentration was negligible. Following supplementation, serum 25(OH)D<sub>2</sub> levels increased in a non-linear fashion from undetectable (< 4 nmol/L) to an estimated mean plateau (95% CI) of 27.1 (20.7, 35.6) nmol/L in the D<sub>2</sub> group (**Figure 4.2**). Using the bootstrap percentile method, the estimated time (95% CI) required to reach 90% of the plateau was 62.9 (29.9, 114.7) d. It should be noted that there was no evidence of a 25(OH)D<sub>2</sub> response in serum by two vitamin D<sub>2</sub>-assigned participants throughout the time course of the study. Both of these participants reported pill compliance between 63–64%. We also found no evidence of a 25(OH)D<sub>2</sub> response in a third participant (83% reported compliance) at the wk 24 visit only. Serum 25(OH)D<sub>2</sub> levels over the time course of the study in the placebo and vitamin D<sub>3</sub> group remained negligible.



**Figure 4.2:** Plot of observed serum 25-hydroxyvitamin D<sub>2</sub> concentration over time in D<sub>2</sub>-treated participants (n=25) and model-estimated line that was fitted to the data with the use of nonlinear regression. The equation is  $y = 27.1 - 26.9e^{-0.03589t}$ ;  $R^2 = 0.437$

**Table 4.2: Total serum 25(OH)D and parathyroid hormone concentrations at baseline and over the 25 wk intervention period<sup>1</sup>**

Measurement and treatment group	Baseline	Week 4	Week 8	Week 12	Week 25	Estimated change after 25 wk after adjusting for baseline <sup>2</sup>
Total serum 25(OH)D(nmol/L)						
Vitamin D <sub>3</sub> (1000 IU/d)	78.8 (73.9, 84.0)	82.7 (77.9, 87.9)	81.9 (76.5, 87.6)	79.6 (73.4, 86.4)	78.9 (71.5, 87.0)	Ref.
Vitamin D <sub>2</sub> (1000 IU/d)	68.3 (60.1, 77.6)	67.2 (59.0, 76.6)	60.1 (52.3, 69.0)	55.9 (47.7, 65.5)	46.5 (37.3, 58.1)	- 22.2 (-14.0, -30.3) nmol/L <sup>2</sup>
Placebo	77.8 (70.5, 85.9)	69.4 (62.7, 76.8)	60.0 (53.5, 67.4)	42.3 (36.6, 49.0)	34.7 (28.5, 42.2)	- 43.6 (-34.8, -50.4) nmol/L <sup>2,3</sup>
Parathyroid hormone (pg/mL)						
Vitamin D <sub>3</sub> (1000 IU/d)	34.0 (29.6, 39.1)	29.7 (25.8, 34.1)	25.8 (22.3, 29.7)	28.0 (23.6, 33.2)	34.6 (29.6, 40.4)	Ref.
Vitamin D <sub>2</sub> (1000 IU/d)	35.9 (30.9, 41.6)	32.3 (28.3, 36.7)	32.4 (27.8, 37.8)	32.2 (26.9, 38.5)	40.8 (34.0, 48.9)	5.4 (-15.8, 31.9) %
Placebo	35.0 (29.8, 41.2)	30.1 (25.7, 35.3)	29.2 (25.4, 33.6)	31.3 (28.5, 34.4)	40.7 (35.6, 46.6)	12.2 (-10.4, 40.6) %

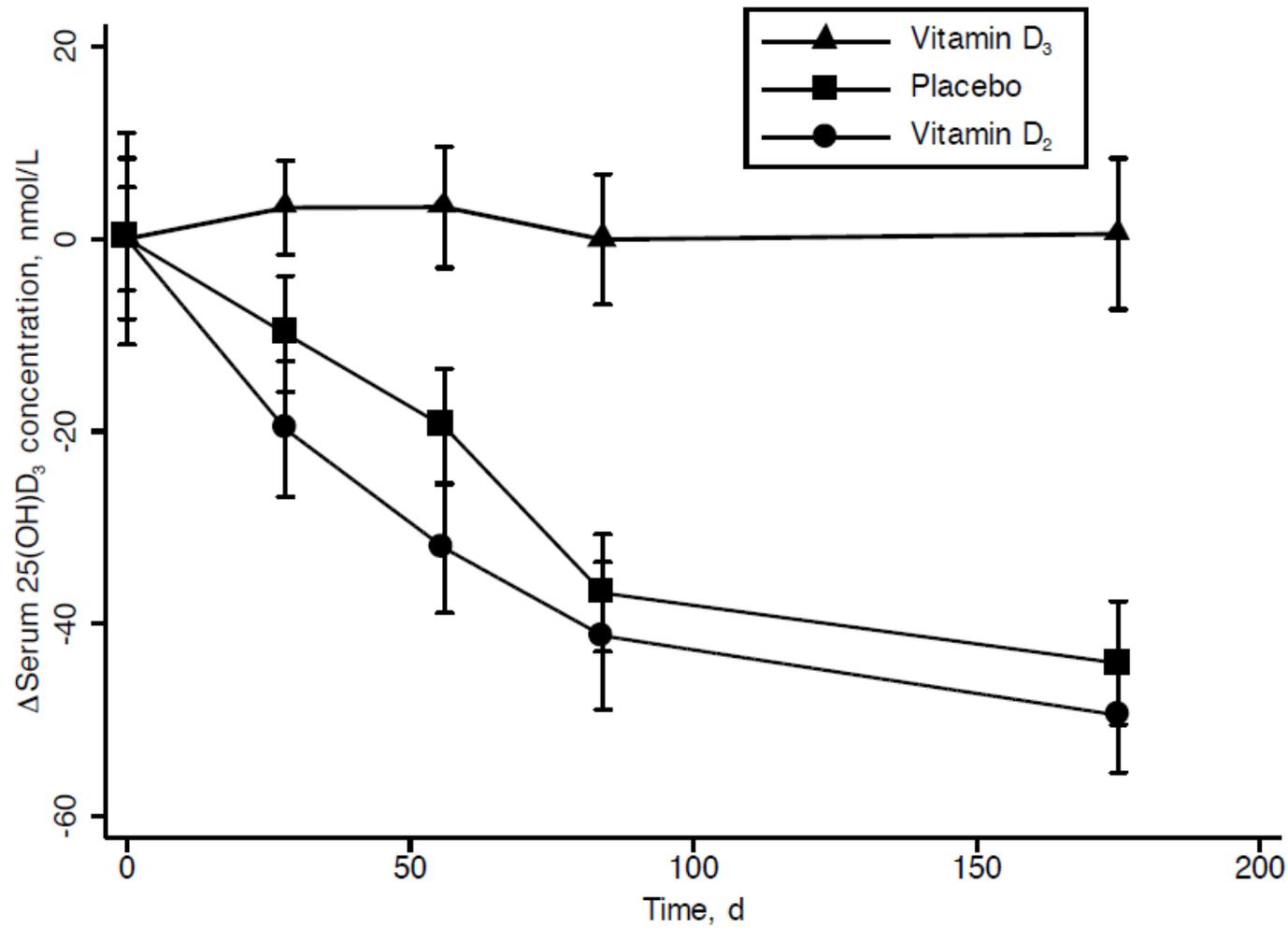
<sup>1</sup> Geometric mean (95% CI).

<sup>2</sup> Significantly different from the vitamin D<sub>3</sub> group after adjustment for baseline concentration,  $P < 0.001$ .

<sup>3</sup> Significantly different from the vitamin D<sub>2</sub> after adjustment for baseline concentration,  $P < 0.001$ .

The mean change in total 25(OH)D [i.e. the sum of 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub>] over the 24 wk intervention period is shown in **Table 4.2**. For total 25(OH)D, adjusting for baseline, there was a statistically significant evidence of a difference in changes between treatment arms ( $P < 0.001$ ). Participants who ingested the 1000 IU vitamin D<sub>3</sub>/d demonstrated no significant change (t-test;  $P = 0.879$ ) in their total 25(OH)D concentrations over the time course of the study whereas mean (95% CI) total 25(OH)D significantly decreased by 43.6 (95% CI 34.8, 50.4) nmol/L ( $P < 0.001$ ) in the placebo group relative to the D<sub>3</sub> group. Despite an increase in serum 25(OH)D<sub>2</sub> among D<sub>2</sub> participants, mean total 25(OH)D concentration decreased significantly relative to the D<sub>3</sub> group reflecting a 22.2 (14.0, 30.3) nmol/L decline over the course of the study ( $P < 0.001$ ). At the end of the study, 24 (80%) and 9 (36%) of participants in the placebo and D<sub>2</sub> group, respectively, had total 25(OH)D concentrations  $< 50$  nmol/L, compared to only 2 (7%) of participants in the D<sub>3</sub> group. Nonetheless, there was no evidence of a statistically significant change in serum PTH concentrations observed between the groups when adjusting for dietary calcium ( $P = 0.355$ ) (**Table 4.2**).

To determine whether intake of supplemental vitamin D<sub>2</sub> had an effect on circulating 25(OH)D<sub>3</sub> concentrations, we evaluated the change over time in 25(OH)D<sub>3</sub> levels among the groups (**Figure 4.3**). Vitamin D<sub>3</sub> treated participants showed no significant change in the serum 25(OH)D<sub>3</sub> throughout the study. As anticipated, both the vitamin D<sub>2</sub> and placebo groups experienced a significant decline in 25(OH)D<sub>3</sub> concentration relative to the vitamin D<sub>3</sub> supplemented group; however, serum 25(OH)D<sub>3</sub>, adjusting for baseline, was significantly lower [7.1 (95% CI 0.8, 13.3) nmol/L] in the vitamin D<sub>2</sub> group compared to the placebo group ( $P = 0.028$ ).



P90

**Figure 4.3:** Mean changes in serum 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] metabolite in participants treated with 1000 IU/d vitamin D<sub>3</sub>, 1000 IU/d vitamin D<sub>2</sub> or placebo over 6 mo intervention. The error bars are 95% CI. After adjustment for baseline, the overall mean change in 25(OH)D<sub>3</sub> values in D<sub>2</sub>-treated participants was 7 nmol/L lower than the control group ( $P = 0.028$ ).

Using a cut-off of 90% compliance, 34 (36%) participants were excluded from the per protocol analysis (11 for unknown compliance; 23 for compliance < 90%), which resulted in a total of 61 participants: 13 in the 1000 IU vitamin D<sub>2</sub>/d, 23 in the 100 IU vitamin D<sub>3</sub>/d and 25 in the placebo. Results (Appendix F) were similar to those from the overall intent-to-treat model; after controlling for baseline 25(OH)D, mean serum 25(OH)D levels decreased by 43.7 (95% CI 37.1, 50.4) nmol/L ( $P < 0.001$ ) in the placebo group relative to the D<sub>3</sub> group. A decrease in total 25(OH)D concentration of 20.8 (12.8, 28.8) nmol/L ( $P < 0.001$ ) was also seen in the vitamin D<sub>2</sub> supplemented group over the 24 week period relative to the D<sub>3</sub>-group. The decline in 25(OH)D<sub>3</sub> concentration observed was also similar to the intent-to-treat analysis. After adjustment for baseline, the overall mean change in 25(OH)D<sub>3</sub> differed between groups (overall  $P < 0.001$ ) with statistically significant decline observed for both the placebo group [44.1 nmol/L (95% CI 37.4, 50.8;  $P < 0.001$ )] and D<sub>2</sub> supplemented participants [52.7 nmol/L (44.5, 60.8;  $P < 0.001$ )] relative to the vitamin D<sub>3</sub> group. Moreover, after adjustment for baseline, the overall mean change in 25(OH)D<sub>3</sub> was greater in participants who received vitamin D<sub>2</sub> [8.6 nmol/L (0.5, 16.6;  $P = 0.036$ )]. No significant differences in PTH concentration were seen for any of the groups, despite controlling for compliance ( $P = 0.809$ ) (Appendix F).

## 4.4 Discussion

Our results show that a daily intake of 1000 IU vitamin D<sub>3</sub> is more effective than 1000 IU vitamin D<sub>2</sub>, as measured by serum 25(OH)D concentration. In New Zealand (latitude ranging from 35°S to 47°S), very few foods are fortified with vitamin D, and the relative contribution of cutaneous production of vitamin D<sub>3</sub> is markedly diminished during the winter months. To our knowledge, this is the first study comparing vitamin D<sub>3</sub> and D<sub>2</sub> by mapping the time course of serum 25(OH)D from the summertime peak through to the wintertime nadir. As expected serum 25(OH)D concentrations decreased substantially among our participants assigned to the placebo group over the course of the study, whereas a daily intake 1000 IU vitamin D<sub>3</sub> was efficacious in maintaining summertime serum 25(OH)D levels. In contrast, while participants assigned to the vitamin D<sub>2</sub> group exhibited significantly higher serum 25(OH)D levels than the placebo group, concentrations were 22 nmol/L (equivalent to approximately 30% of the baseline mean) lower at the end of the study relative to the vitamin D<sub>3</sub> group. These results remained consistent when only those with 90% compliance were included in the analysis, strengthening the study findings. Furthermore, it appeared that the intake of vitamin D<sub>2</sub> had a negative effect on circulating 25(OH)D<sub>3</sub> status, which may partly explain the overall lack of ability of vitamin D<sub>2</sub> to maintain serum 25(OH)D levels. Nonetheless, despite differences in 25(OH)D status among the three treatment groups, there was no evidence of a difference in PTH concentrations.

These data are in agreement with previous observations using higher dosing regimens. Trang et al. (1998) and Tjellesen et al. (1986), both whom used 4000 IU/d, found that vitamin D<sub>2</sub> was less effective than vitamin D<sub>3</sub> in increasing serum 25(OH)D levels.

Similarly, Armas et al. (2004), Heaney et al. (2011) and Binkley et al. (2011) found substantial discrimination in favour of D<sub>3</sub> using either a single, weekly or monthly doses of 50,000 IU. Furthermore, our findings of a greater decline in serum 25(OH)D<sub>3</sub> levels in the D<sub>2</sub>-treated participants than the placebo group has also been previously reported (Armas et al., 2004; Binkley et al., 2011). There are several possible mechanisms that may explain the lack of an equivalent increase in total 25(OH)D in vitamin D<sub>2</sub> supplemented groups. It has been proposed that an up-regulation in mechanisms required to metabolise vitamin D<sub>2</sub> and its metabolites may increase the metabolic degradation of circulating 25(OH)D<sub>3</sub> levels (Armas et al., 2004). Binkley and colleagues (2011) have suggested that metabolic differences related to substrate competition for the 25-hydroxylase enzyme may explain the decrease in 25(OH)D<sub>3</sub> concentration following dosing with vitamin D<sub>2</sub>. Furthermore, serum 25(OH)D<sub>2</sub> has been shown to have a less avid binding affinity for the carrier protein, vitamin D binding protein (DBP), than does 25(OH)D<sub>3</sub> (Hollis, 1984), which may result in a shorter body half-life of 25(OH)D<sub>2</sub>.

The only randomised controlled trial demonstrating effective equivalence of vitamin D<sub>2</sub> and D<sub>3</sub> was conducted by Holick et al. (2008), whom reported that serum 25(OH)D levels increased to the same extent in participants receiving 1000 IU daily as vitamin D<sub>2</sub>, vitamin D<sub>3</sub> or a combination of 500 IU vitamin D<sub>2</sub> and 500 IU vitamin D<sub>3</sub>. The results of this study suggested that the pharmacokinetic parameters of vitamin D<sub>2</sub> and vitamin D<sub>3</sub> change with increasing dose, i.e. a disproportionate rise in serum levels between vitamin D<sub>2</sub> and D<sub>3</sub> such that low doses of D<sub>2</sub> and D<sub>3</sub> appear equivalent while higher doses of D<sub>2</sub> are less effective than D<sub>3</sub>. Several limitations, however, should be noted when reviewing this previous study. First, the detection limit for the assay used was 10 nmol/L, and values for serum 25(OH)D<sub>2</sub> less than 10 nmol/L were obtained by subtracting 25(OH)D<sub>3</sub> from the total 25(OH)D. Given

both the analytical and biological variability in serum 25(OH)D, the method employed to quantify serum 25(OH)D<sub>2</sub> levels would likely result in a considerable overestimation of the actual concentrations. In contrast, our assay was more sensitive with a non-detectable serum 25(OH)D<sub>2</sub> level of less than 5 nmol/L. Furthermore, we assumed a value of zero for any 25(OH)D reported as less than 5 nmol/L. In addition, participants in Holick's study (Holick et al., 2008) were generally obese (mean BMI of 30 kg/m<sup>2</sup>), which may have an effect on the outcome measure of total serum 25(OH)D; and nearly 40% (6 out of 16) participants assigned to D<sub>2</sub> group were taking a 400 IU vitamin D<sub>3</sub>-containing supplement during the study. We sought to avoid the effects of potential predictors in our study with the inclusion criteria requiring participants to have a BMI of less than 25 kg/m<sup>2</sup> and prohibited the use of vitamin D-containing supplements.

The Food and Nutrition Board of the Institute of Medicine has recommended 400 IU vitamin D daily to meet the needs of half of adults up to the age of 50 y, and 600 IU daily to meet the needs of 97.5% of these adults (Institute of Medicine, 2010). In our study, a daily intake 1000 IU of vitamin D<sub>3</sub> maintained summer 25(OH)D levels. Using data from controlled trials, a regression analysis of the relationship between serum 25(OH)D level and total intake of vitamin D predicts that a daily intake of 1000 IU would be associated with a mean serum 25(OH)D level of 68 nmol/L [ $y = 9.9 \ln(\text{total vitamin D intake})$ ] (Institute of Medicine, 2010). The predicted level is noticeably less than the present study mean level of 79 nmol/L achieved at the end of the study in our D<sub>3</sub>-treated participants; however, it should be noted that the simulated intake-response relation has been determined under conditions of minimal sun exposure, which may not be fully met at latitudes below 49° (Institute of Medicine, 2010). In contrast, the predicted value is substantially higher than the mean serum

25(OH)D level of 47 nmol/L observed in our D<sub>2</sub> participants. Neither the simulated intake-response relation nor the newly revised DRI distinguish between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>.

We did not measure the active form of 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] metabolites of the two vitamin D forms. It is not known whether the lower level of circulating 25(OH)D observed in our D<sub>2</sub>-treated participants would result in lower production of the di-hydroxylated form. The synthesis of adequate 1,25(OH)<sub>2</sub>D production is dependent on the level of the serum 25(OH)D precursor and the enzyme 25-hydroxyvitamin D-1- $\alpha$ -hydroxylase (also known as CYP27B1), which converts 25(OH)D to 1,25(OH)<sub>2</sub>D (Morris & Anderson, 2010). The expression of renal CYP27B1 is tightly regulated and plays an essential role in maintaining calcium and phosphate homeostasis. When serum 25(OH)D levels fall, there is a rise in parathyroid hormone, which in turn up-regulates the production of the renal CYP27B1 enzyme. In our study, we found no evidence of a difference in parathyroid hormone concentration despite appreciable differences in serum 25(OH)D concentrations among treatment groups. Given the strong interdependence of vitamin D and calcium, it is speculated that the relatively high dietary calcium intakes in our study population may have suppressed the rise in serum PTH that typically accompanies declining 25(OH)D concentrations (Seamans & Cashman, 2009; Steingrimsdottir et al., 2005; Weaver & Fleet, 2004). As a result, 1 $\alpha$ -hydroxylation may be reduced causing a fall in renal synthesis of 1,25(OH)<sub>2</sub>D. Although knowledge of the regulation of CYP27B1 expression in other tissues such as skeletal muscle, liver and lung is limited, non-renal CYP27B1 may be unable to up-regulate under conditions of low circulating 25(OH)D causing an earlier decline in tissue-specific 1,25(OH)<sub>2</sub>D levels than plasma (Morris & Anderson, 2010). However, the absence of changes in physiological endpoints such as PTH concentration and bone turnover

markers led Binkley et al. (2011) to question whether the lesser potency of vitamin D<sub>2</sub> has biological implications.

In conclusion, daily administration of 1000 IU vitamin D<sub>3</sub> over 24 wk intervention period was more effective as similarly administered vitamin D<sub>2</sub> in maintaining serum 25(OH)D levels. These findings contribute to the accumulating evidence that vitamin D<sub>3</sub> and vitamin D<sub>2</sub> have different pharmacokinetic profiles for serum 25(OH)D. The assumption of vitamin D<sub>2</sub> and D<sub>3</sub> equivalence from both a physiological and a therapeutic basis should be re-examined particularly given that the dose employed in our study is commonly used in over-the-counter dietary supplements. Nonetheless, conclusions about the biological significance of the different effects of the two forms cannot be drawn, and more studies are needed to determine whether lower doses would also suggest differences in pharmacokinetic parameters between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>.

## 5 Concluding Comments

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### 5.1 Conclusion

The mean serum 25-hydroxyvitamin D [25(OH)D] concentration of this group of healthy adults from Dunedin, New Zealand (latitude 45°S) recruited at the end of summer was 75 nmol/L. Only 7% of participants were at risk of inadequate vitamin D status [25(OH)D < 50 nmol/L] suggesting that the majority of the sample had sufficient vitamin D levels. The only predictor of baseline vitamin D status in this study was the use of a vitamin D-containing supplement or multi-vitamin in the three months prior to commencing the study.

The decline in 25(OH)D concentration seen in the placebo group over the study duration (winter period) was expected, as a seasonal variations in vitamin D status have been previously reported in the New Zealand population, reflecting the lack of cutaneous synthesis occurring at this time. However, a 1000 IU Vitamin D<sub>3</sub> supplement, administered daily for 6 months, maintained serum 25(OH)D concentrations over the winter period in Dunedin. Vitamin D<sub>2</sub> potency was less than that of vitamin D<sub>3</sub>, as supplementation with an equivalent dose of vitamin D<sub>2</sub> was not as effective at maintaining serum 25(OH)D. Furthermore, although vitamin D<sub>2</sub> supplements lessened the decline in total 25(OH)D concentration over winter (as seen in the placebo group), there was a decline in circulating 25(OH)D<sub>3</sub> concentration, without an equivalent increase in circulating 25(OH)D<sub>2</sub> concentration. The similarity

of the results from both the intention-to-treat and per protocol analysis lends further strength to these findings. Thus, the main conclusion that can be drawn from this study is that vitamin D<sub>3</sub> (cholecalciferol) and vitamin D<sub>2</sub> (ergocalciferol) are not biologically equivalent forms.

## **5.2 Strengths and limitations of study design**

### **5.2.1 Strengths**

Several aspects of our study lend weight to the findings. This was a double-blinded, randomised, placebo controlled trial, essentially the gold standard for quality of clinical evidence as it limits information, selection and confounding bias (Grimes & Schulz, 2002). Previous studies undertaken in this area have been limited by confounding variables and insufficient duration and have been lacking in statistical power (sample size) to detect a difference. Thus a major strength was the study design, which limited the impact of confounding variables by controlling for season, BMI and age. The study was conducted in New Zealand at a latitude of 45°S where food fortification of vitamin D is neither mandated nor common. Participants also discontinued taking vitamin D-containing supplements prior to commencing the study, and thus the outcome was not affected by background dietary intake of vitamin D. The study was sufficiently powered to detect a difference in the primary outcome, and was of sufficient duration to investigate the long-term effect of supplementation on 25(OH)D concentration, which has been reported to plateau between six weeks to six months (Heaney et al., 2003a; Holick et al., 2008; Smith et al., 2009). In addition, the anthropometric measurements were all carried out according to standardised procedures, and the follow-up questionnaire at each blood draw monitored any changes that may confound the study results (i.e. changes in medication, supplement intake and overall health).

The isotope-dilution liquid chromatography tandem mass spectroscopy method used to analyse serum 25(OH)D concentration is highly accurate (Maunsell et al., 2005), and enabled both 25(OH)D<sub>2</sub> and 25(OH)D<sub>3</sub> concentrations to be determined separately. Subsequently, the study was able to assess differences in the effect of the two forms on serum 25(OH)D concentrations, providing accurate information regarding the potencies of the two supplement forms. The analysis of serum 25(OH)D was completed to a high standard, with one international reference control and one internal serum sample. A further strength was the measurement of PTH concentration over the course of the study, thus the effect of the different forms of vitamin D on a functional indicator of vitamin D status could be observed. Compliance was also high (93%) and relatively few participants dropped out of the study (11%).

### **5.2.2 Limitations**

The majority of the limitations occurred around the determination of indicators of vitamin D status at baseline, as the study population was rather homogeneous. Most participants were New Zealand Europeans, with light facultative skin colour, limiting the ability to see an association between skin colour and vitamin D status. All participants had normal BMI (19–25 kg/m<sup>2</sup>) and were aged 18–50 years, thus an affect of BMI and age on vitamin D status was less likely to be observed due to the narrow range of each variable within the study population.

Differences in compliance amongst the three groups may be a limitation, as there was lower overall compliance in the vitamin D<sub>2</sub> supplemented group, as demonstrated by the per protocol analysis. This may affect interpretation of the degree

of variation in efficacy of vitamins D<sub>2</sub> and D<sub>3</sub> at improving vitamin D status, nevertheless the two remained significantly different in the per protocol analysis.

The high calcium intake of participants may also be a limitation, as this influenced the ability to determine seasonal variation and the effect of the two forms of vitamin D on serum PTH concentrations. Furthermore, the assessment of dietary calcium intake in this study was in itself a limitation in this study. Although a seven-day diet record is the recommended method of assessing dietary calcium intake (Basiotis et al., 1987), only 56% of participants returned their baseline diet records. The software program used to analyse dietary calcium intakes used food composition data from 1998, thus may inaccurately represent the current calcium content of some foods available at this time.

### **5.3 Implications and Future Research Directions**

The finding that the only significant predictor of vitamin D status in this population of non-obese adults aged < 70 years was the use of a vitamin D-containing supplement shows that even in small doses, supplemental vitamin D is an important source of vitamin D in New Zealand. Furthermore, the high risk of melanoma skin cancer associated with sun exposure coupled with insufficient UV synthesis of vitamin D during wintertime reinforces the need to increase the availability of vitamin D supplements to New Zealanders (with high dose supplementation for high risk groups such as the elderly).

Considerable research has been undertaken assessing determinants of vitamin D status in different populations, however the relationship between constitutive and facultative skin colour and 25(OH)D concentration remains complex. Further research examining this relationship in those with a wide range of constitutive and facultative skin colours would be useful in determining the impact of both melanin and sunlight exposure on vitamin D status. A longitudinal study evaluating change in skin colour and sunlight exposure of the summer period and the affect on 25(OH)D concentration would provide valuable insight into these predictors of vitamin D status. This information may be useful for clinicians when assessing who may be at risk of insufficient or deficient vitamin D levels. It would also have implications for public health guidelines regarding sun exposure and the need for vitamin D supplements.

Furthermore, the finding that the two forms of vitamin D are not equivalent has implications for countries where there is widespread fortification of foods with vitamin D but no specification regarding the form of vitamin D used. The prescription of vitamin D supplements by General Practitioners is also affected, as the effect of high dose vitamin D<sub>2</sub> supplements may not produce the desired outcome of improved 25(OH)D concentrations. In New Zealand, where the prevalence of vitamin D insufficiency is high, especially during winter, the only way to improve vitamin D status safely and effectively is through increased fortification of foods with vitamin D and/or the administration of vitamin D supplements. Thus, the Dietary Reference Intakes for vitamin D should take supplemental form into considerations when making recommendations regarding the use of supplements and the Adequate Intake levels for population groups in New Zealand. The differences in efficacy between the two vitamin D forms also has implications for future research undertaken that includes vitamin D, e.g. in determining the role of vitamin D status in chronic disease risk. When designing trials and assessing the results of studies the type of supplemental vitamin D used, and ensuing effect, needs to be considered.

It is unknown whether the difference in efficacy of the two forms results in different biological effects, seeing that despite the decline in total 25(OH)D concentration in the placebo and vitamin D<sub>2</sub>-supplemented groups there was no change in PTH concentration. The study was not primarily designed to compare the effect of the two vitamin D forms on PTH concentration. It would therefore be of interest to investigate the differences in response to supplementation with vitamin D<sub>2</sub> or vitamin D<sub>3</sub> in participants with vitamin D deficiency, as this would provide insight

into potential biological implications. (However, research of this nature may be unethical.) In determining whether there are potential biological implications, it would be valuable to investigate whether there are corresponding changes in concentration of 1,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D] metabolites of the two vitamin D forms. It is not known whether lower levels of circulating 25(OH)D following supplementation with vitamin D<sub>2</sub> results in lower production of the dihydroxylated form.

Additional studies are needed to determine whether lower doses of vitamin D supplements also suggest differences in pharmacokinetic parameters between vitamin D<sub>2</sub> and vitamin D<sub>3</sub>. This is important as fortification of food with vitamin D and over-the-counter supplements generally use much lower doses of vitamin D. At low doses there may be less substrate competition or up-regulation of mechanisms for vitamin D metabolism, thus the impact of vitamin D<sub>2</sub> supplementation on circulating levels of 25(OH)D<sub>3</sub> may not be as apparent at low doses.

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## 7 Appendices

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Appendix A: Study Advertisement

Appendix B: Baseline Questionnaire

Appendix C: Information Sheet and Consent Form

Appendix D: 7-day Diet Record

Appendix E: Follow-up Questionnaire

## **7.1 Appendix A: Study Advertisement**

Worried about the lack of sunlight in winter affecting your bones???

Our bodies make Vitamin D when they are exposed to sunlight. Low levels of Vitamin D have been associated with bone fractures, heart disease, high blood pressure and diabetes.

The Department of Human Nutrition at the University of Otago is looking for volunteers to participate in a study to find out if taking Vitamin D and Calcium supplements over the winter can help maintain and improve the levels of vitamin D in your blood.

You will Receive \$100 for participating.

If you are aged 18-50, will be staying in New Zealand over the winter, and would like further information please contact:

Meredith Rose  
479 5673  
email: [vitamind@otago.ac.nz](mailto:vitamind@otago.ac.nz)

## **7.2 Appendix B: Baseline Questionnaire**

# Vitamin D Supplementation Study

University of Otago  
Department of Human Nutrition

Co-ordinators

Meredith Rose

Dr Lisa Houghton  
Dr Murray Skeaff



*The aim of this questionnaire is to collect general information on the participants taking part in this Supplementation Study.*

*All information you give is strictly confidential.  
No one outside of the study will have access to this information.*

**Thank you for participating in this research study.  
We appreciate the time you are giving.**

Full Name: \_\_\_\_\_

Address: \_\_\_\_\_

\_\_\_\_\_

Phone: \_\_\_\_\_

Cell: \_\_\_\_\_

Email: \_\_\_\_\_

Preferred  
method of Contact: \_\_\_\_\_

1. What is your date of birth? \_\_\_\_\_

2. Are you:

Male

Female

3. Which ethnic group or groups do you belong to? (Tick all that apply)

NZ European

Maori

Samoan

Cook Island

Tongan

Niuean

Chinese

Indian

Other such as Dutch, Japanese, Tokelauan.

**Please state** \_\_\_\_\_

4. What is your highest level of education that you have completed?

Less than highschool

Highschool graduate

Some university/tertiary study

Certificate or Diploma

University degree

Advanced degree.

**Please state** \_\_\_\_\_

5. How would you describe your health?

- EXCELLENT
- GOOD
- FAIR
- POOR

6. Have you had any of the following medical conditions?

- Liver Disease
- Kidney Disease
- Other Health Problems

Please describe.

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7. Are you currently taking, or have you taken in the last three months, a dietary supplement containing Calcium?

- NO
- YES

IF yes please specify below:

Brand of Supplement: \_\_\_\_\_

Amount of Calcium (if known): \_\_\_\_\_

Date last taken (approx): \_\_\_\_\_

8. Are you currently taking, or have you taken in the last three months, a dietary supplement containing Vitamin D?

- NO
- YES

IF yes please specify below:

Brand of Supplement: \_\_\_\_\_

Amount of Vitamin D (if known): \_\_\_\_\_

Date last taken (approx): \_\_\_\_\_

9. Do you use, or have you used in the last three months, sunless tanning lotion on your arms?

NO

YES

IF yes please specify below:

Brand of lotion: \_\_\_\_\_

Frequency of use (e.g. daily): \_\_\_\_\_

Date last used (approx): \_\_\_\_\_

10. Do you take any other prescribed medication (*including oral contraceptives*) on a **regular** basis?

NO

YES

IF yes, please specify below:

(i) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

(ii) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

(iii) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

11. Are you planning on taking a holiday outside New Zealand between March and August 2009?

NO

YES

IF yes, please specify below:

(i) Location: \_\_\_\_\_

Length of stay: \_\_\_\_\_

(ii) Location: \_\_\_\_\_

Length of stay: \_\_\_\_\_

### **7.3 Appendix C: Information Sheet and Consent**

[Reference Number as allocated upon approval by the Ethics Committee]

[Date]

## **Effect of vitamin D2 and D3 on Serum 25-hydroxyvitamin D and parathyroid concentrations in health adult subjects**

### **INFORMATION SHEET FOR PARTICIPANTS**

Thank you for showing an interest in this project. Please read this information sheet carefully before deciding whether or not to participate. If you decide to participate we thank you. If you decide not to take part there will be no disadvantage to you of any kind and we thank you for considering our request.

#### **What is the Aim of the Project?**

Vitamin D plays an important role in calcium absorption and bone health. Vitamin D can only be obtained in small amounts in the diet, and most of our body's vitamin D is produced in our skin when it is exposed to sunlight. In New Zealand at least 40% of all children and adults are vitamin D deficient. This deficiency is due partly to an increased use of sun protection, but mostly because UVB from the sun is not strong enough during the winter months in New Zealand to generate production of vitamin D in the skin. The only way to improve vitamin D nutrition effectively and safely is to add extra vitamin D to the foods we eat, or to take a dietary supplement.

The aim of this project is to compare how the two different types of vitamin D (vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol)) affect the levels of vitamin D (serum 25-hydroxyvitamin D) and parathyroid hormone (a functional biomarker of vitamin D levels) in your blood over the first 4 months of autumn and winter.

#### **What Type of Participants are being sought?**

We are seeking healthy adults between the ages of 18 and 50 years. You cannot take part in this study if you are currently taking a vitamin D supplement and don't want to stop taking for the study period, or if you are planning a holiday north of x but south of y between February and June 2009 (as this will increase the production of Vitamin D in your skin). Furthermore, people who are in one or more of the categories listed below will not be able to participate in the project because, in the opinion of the researchers and the University of Otago Human Ethics Committee, it may involve an unacceptable risk to them:-

- Individuals with chronic liver and kidney disease
- Individuals who are taking medication that might affect vitamin D metabolism, including anticonvulsants, glucocorticoids and barbituates.
- Individuals with known chronic disease such as Crohns, Ulcerative Colitis, Diabetes and Cardiovascular disease

#### **What will Participants be Asked to Do?**

Should you agree to take part in this project, you will be asked to attend ten (10) clinic visits at the university over a five-month period. At the first clinic visit your height and weight will be measured, you will be given a questionnaire about your multivitamin and dairy consumption. You will then be provided with a months supply of calcium supplements to ensure your calcium intake is adequate before beginning the Vitamin D study. The second visit occur a month later, at which a blood sample will be taken (xx mL???) and you will be randomly assigned into one of the following supplement groups: (1) 1000 international units of vitamin D2, (2) 1000 international until of vitamin D3, or (3) a placebo (no vitamin D). The researchers and you will not know which supplement you have been assigned until the end of the study. You will be instructed to take your supplement on a daily basis for the next four months.

Over the next four months we will ask that you maintain your regular diet, but to avoid the intake of supplements containing vitamin D. You will be asked to return to the clinic eight more times over the four month period (more frequently over the first two months, and less frequently over the second four months) for blood samples to be taken. Each visit will take no more than 30 minutes.

#### **Potential Harm of Discomfort**

There are no known adverse effects associated with taking supplements containing vitamin D. You may experience slight pain during the drawing of blood samples, and in some cases minor bruising after the sample has been taken, but this generally disappears in about one to two days. A registered nurse will draw your blood and a light refreshment (tea, coffee, juice, biscuits) will be provided to you at the completion of your visit.

#### **Can Participants Change their Mind and Withdraw from the Project?**

You may withdraw from participation in the project at any time without any disadvantage to yourself of any kind.

#### **What Data or Information will be Collected and What Use will be Made of it?**

We will be collecting personal information regarding your age ethnicity, weight, height, medical history, alcohol and tobacco use, as well as prescription medicines you are currently taking.

The purpose of collecting this information is so that we are able to describe the overall characteristics of the study population. Only Lisa Houghton and Murray Skeaff will have access to personal information and even then only ID number will identify study individuals. The data collected will be securely stored in such a way that only those mentioned above will be able to gain access to it. At the end of the project any personal information will be destroyed immediately, except that, as required by the University's research policy, any raw data on which the results of the

research project depend will be retained in secure storage for five years, after which it will be destroyed.

Group results of this project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but any data included will in no way be linked to any specific participant. You are most welcome to request a copy your personal results, along with a brief written interpretation of them, following the end of your participation in the study and/or a copy of the results of the project should you wish.

### **What if Participants have any Questions?**

If you have any questions about our project, either now or in the future, please feel free to contact either:-

Meredith Rose

or

Lisa Houghton

Department of Human Nutrition

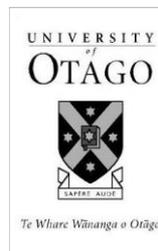
Department of Human Nutrition

University Telephone Number:- 479 5673

University Telephone Number:- 479

7294

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph 03 479 8256). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.



## Effect of vitamin D2 and D3 on Serum 25-hydroxyvitamin D and parathyroid concentrations in health adult subjects

### CONSENT FORM FOR PARTICIPANTS

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:-

1. My participation in the project is entirely voluntary;
2. I am free to withdraw from the project at any time without any disadvantage;
3. Personal identifying information will be destroyed at the conclusion of the project but any raw data on which the results of the project depend will be retained in secure storage for five years, after which they will be destroyed;
4. I am aware that I may experience slight pain during the drawing of blood, and in some cases minor bruising may occur but this will generally disappear in about one to two days;
5. I am aware that at the end of the study I will receive \$100 for the compensation of costs associated with travel to and from the clinic
6. The results of the project may be published and will be available in the University of Otago Library (Dunedin, New Zealand) but every attempt will be made to preserve my anonymity.

I agree to take part in this project.

.....  
(Signature of participant)

.....  
(Date)

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph 03 479 8256). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.

## **7.4 Appendix D: 7-day Diet Record**

# Vitamin D Supplementation Study

# 7-Day Diet Record

University of Otago  
Department of Human Nutrition



## Instructions

We would like you to record in the booklet EVERYTHING you eat or drink on for 7 days of the next 2 weeks (including 2 weekend days and 5 weekdays).

Writing down everything that you eat and drink can be inconvenient but please try not to change what you consume because you are keeping a record.

### Describing foods and Drinks

The more detail you are able to give about the food and drink you have consumed, the better we are able to estimate your nutrient intake.

- Please record the **brand** name of each food, drink or cooking ingredient wherever possible. If you think the ingredient is unusual, please feel free to staple the wrapper to this booklet.
- Please describe each item, including **cooking** details and any **salt, sugar, spices and sauces** you have added before eating.

e.g. Chicken Breast

Tegal

with fat, bone and skin removed. Fried in  $\frac{1}{2}$  tsp canola oil

## Estimating the quantity of food or drink consumed

Because describing amounts can be difficult, here are some tips to make it easier for you to estimate the amount of each food or drink you consume.

- Use Household measures  
e.g. 2 rounded teaspoons of white sugar  
or 1 cup of Uncle Bens rice
- Record weight marked on packages  
e.g. 150g Fresh n Fruity yoghurt
- Compare to other objects  
e.g. Tasty cheese, size of a match box
- Use a ruler, or estimate the size  
e.g. Orange, peeled 10cm in diameter

For mixed food dishes it may be easier to list the total ingredients on another piece of paper, then describe the proportion of this recipe you consumed

## Ingredient lists for recipes

On a separate piece of paper please record the recipe, including brand names, amounts and preparation or cooking details. Then on your diet record sheet indicate the proportion on the recipe you consumed.

e.g. On a Separate piece of paper record:

### Creamy tuna pasta (recipe 1)

250g	Diamond spiral pasta
$\frac{1}{2}$ cup	Oxo chicken stock, 1 teaspoon on stock pre-mixed with water
$\frac{1}{4}$ cup	Chopped parsley
2 cups	Sliced button mushrooms
220g	John West canned in oil Tuna, liquid drained
1 cup	Carnation evaporated skim milk
1 tablespoon	Parmesan Cheese, dried
$\frac{1}{4}$ teaspoon	freshly ground black pepper

On the appropriate days diet record sheet record:

Under Food or Drink write: **Recipe 1**

Under Quantity record the proportion of the recipe you ate: **1/3 recipe**

Sample record sheet

Please record **ALL** food and drink consumed during the whole day, including snacks and water.

Remember to report any **ADDITIONS** to each food or drink such as milk, sugar, sauce or spreads.

Time	Food or Drink	Brand and Details	Preparation/Cooking	Quantity
10am	Muffin	Bought - Banana		6 cm diameter, 9 cm tall
	Coffee	Bought - Cappuccino		Large cup
12pm	Creamy tuna pasta	Homemade- recipe 1		1/3 recipe
	French bread stick	Bought - New World		6 cm diameter, 6 cm long
	Margarine	Pams -Canola low salt		2 tablespoons
	Chicken breast	Skin and bone removed	Fried in oil	76 gm
	Olive oil	Luppi	fried	$\frac{1}{2}$ tbsp
	Tomatoes		raw	2 tomatoes 5 cm diameter
	Spring onions		raw	1
	Vinegar	Red wine		1 tbsp
	Banana			16 cm long
	Orange juice	McCoy, unsweetened		200 mL

Record **brand names**. e.g. McCoy

Use **household measures** to describe amounts of foods such as margarine, butter and milk e.g. teaspoon (tsp), tablespoons (tbsp), cups (cp)

Use a **ruler** to estimate the dimensions e.g. French bread stick, 6 cm diameter, 6 cm long





























## **7.5 Appendix E: Follow-up Questionnaire**

# Vitamin D Supplementation Study

Date: \_\_\_\_\_

1. Have you used sunless tanning lotion (fake tan) on your arms or forehead in the last 12 weeks?

NO

YES

IF yes please specify below:

Brand of lotion: \_\_\_\_\_

Frequency of use (e.g. daily): \_\_\_\_\_

Date last used (approx): \_\_\_\_\_

2. Has your prescribed medication (***including oral contraceptives***) changed over the last 12 weeks?

NO

YES

IF yes, please specify below:

(i) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

(ii) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

(iii) Name: \_\_\_\_\_

Dose: \_\_\_\_\_

Length of time on medication: \_\_\_\_\_

3. Have you been on holiday outside of Dunedin in the last 12 weeks?

NO

YES

IF yes, please specify below:

(i) Location: \_\_\_\_\_

Length of stay: \_\_\_\_\_

(ii) Location: \_\_\_\_\_

Length of stay: \_\_\_\_\_

4. Have you taken your study supplement almost every day since your last visit?

NO

YES

IF NO, please explain: \_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

*[The following questions were asked at the final visit only]*

5. Which supplement group do you think you were in? \_\_\_\_\_

Why? \_\_\_\_\_

6. Do you have any comments regarding this study, or suggestions to improve it?

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

7. Would you be interested in participating in other studies?

NO

YES

IF yes, please provide your email address: \_\_\_\_\_

**Smoking questions (asked post-intervention).**

1. Do you smoke?

If yes, how many cigarettes per day?

2. Have you smoked in the past?

If yes, when did you stop smoking?

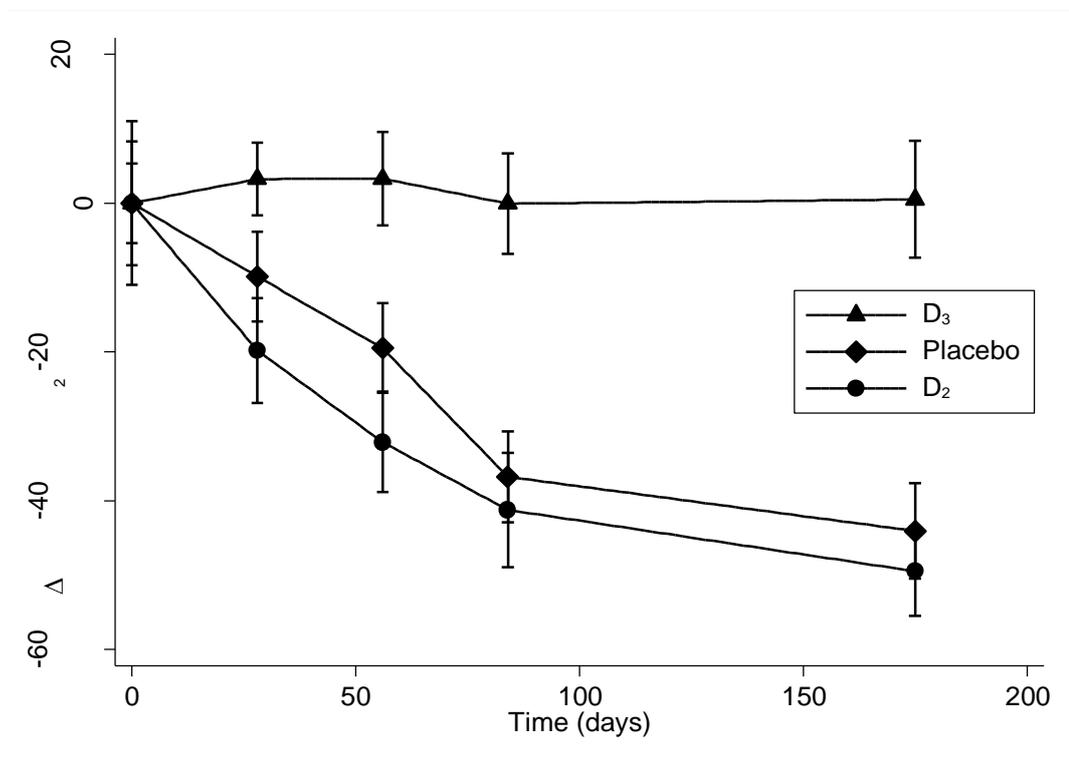
3. Do you live with someone who smokes?

If yes, please describe their smoking habit, e.g. do they smoke inside or outside, how many cigarettes/day?

## 7.6 Appendix F: Per Protocol Analysis

**Table 2.** Total serum 25(OH)D and parathyroid hormone concentrations at baseline and over the 25 wk intervention period<sup>1</sup>

Measurement and treatment group	Baseline	Week 4	Week 8	Week 12	Week 25	Estimated change after 25 wk after adjusting for baseline <sup>2</sup>
Total serum 25(OH)D(nmol/L) n=61						evidence of difference in change between groups p<0.001
Vitamin D <sub>3</sub> (1000 IU/d)	79.8 (74.5, 85.1)	83.0 (78.1, 88.0)	83.1 (76.8, 89.3)	79.7 (73.0, 86.4)	80.3 (72.4, 88.1)	ref
Vitamin D <sub>2</sub> (1000 IU/d)	74.0 (62.8, 85.2)	70.0 (62.5, 77.5)	65.7 (59.2, 72.2)	61.8 (54.2, 69.4)	56.4 (49.8, 62.9)	-20.8 (-12.8, -28.8) p<0.001
Placebo	80.5 (72.1, 89.0)	70.9 (64.8, 77.0)	61.3 (55.2, 67.4)	43.7 (37.7, 49.8)	36.9 (30.4, 43.4)	-43.7 (-37.1, -50.4) p<0.001
Parathyroid hormone (pg/mL) n=61						no evidence of difference in change between groups p=0.809
Vitamin D <sub>3</sub> (1000 IU/d)	36.9 (32.1, 41.7)	34.0 (29.2, 38.8)	29.2 (25.0, 33.3)	32.2 (27.2, 37.2)	40.1 (33.7, 46.4)	ref
Vitamin D <sub>2</sub> (1000 IU/d)	38.4 (29.6, 47.2)	32.7 (26.2, 39.2)	34.5 (27.4, 41.6)	33.1 (27.2, 39.1)	43.3 (35.0, 51.5)	+8.4% (-13.7%, +36.0%)
Placebo	37.6 (31.2, 44.0)	32.3 (28.0, 36.6)	31.8 (25.8, 37.8)	32.0 (28.6, 35.5)	43.4 (37.3, 49.5)	+10.2% (-8.8%, +33.1%)



**Figure 2.** Unadjusted mean changes in serum 25-hydroxyvitamin D<sub>3</sub> [25(OH)D<sub>3</sub>] metabolite in participants treated with 1000 IU/d vitamin D<sub>3</sub>, 1000 IU/d vitamin D<sub>2</sub> or placebo over 6 mo intervention (n=61). The error bars are 95% CI.