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Effects of bolus dose vitamin D₃ on vitamin D metabolites, calcium metabolism, bone turnover markers, physical function and cardiovascular parameters in post-menopausal women

Simon David Bowles

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The University of Sheffield
Faculty of Medicine, Dentistry & Health
Department of Oncology & Metabolism

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Abstract

Several studies have shown an increase in the rate of falls and fractures after high dose vitamin D supplementation but the mechanism for the increase in falls and fracture is yet to be elucidated. One proposed mechanism is that the binding capacity of VDBP can become saturated in some situations (such as after a single large bolus dose). Therefore, the increased rate of falls and fractures that have been previously reported after a single large bolus dose of vitamin D may have been due to vitamin D toxicity in the initial period after administration due to the binding capacity of VDBP being overwhelmed by the large influx of vitamin D into circulation. It has been hypothesised that this may lead to a sharp increase in total 25(OH)D with a relatively greater increase in free 25(OH)D. In addition, there may also be a disproportionate rise in free 1, 25(OH)₂D, because this metabolite has the weakest binding affinity for VDBP. There may then be several consequences of disproportionate increases in one or more of these free vitamin D metabolites that may increase the propensity for people to fall, such as hypercalcemia. Vitamin D is a potent suppressor of renin synthesis and excess free metabolites could impact upon the renin-angiotensin system, leading to postural hypotension and possibly leading to falls. Elevated free metabolites may also have direct effects on the brain (the VDR is intensely expressed in the cerebellum which is heavily involved in balanced muscular activity) and/or direct effects on muscle.

This study set out to investigate the clinically important hypothesis that a disproportionate rise in free vitamin D metabolites and hypercalcemia could explain the increase in falls and fractures after high dose vitamin D that have been previously reported.

The study was a single centre, double-blinded, randomised, controlled trial carried out at the Clinical Research Facility (Northern General Hospital, UK) to determine the effects of three different oral bolus doses of vitamin D₃ (50 000IU, 150 000IU and 500 000IU) on total and free 25(OH)D and total and free 1, 25(OH)₂D in vitamin D deficient, but otherwise healthy, postmenopausal women. Thirty-three vitamin D deficient (25(OH)D <30nmol/l) postmenopausal women were randomized to one of the three treatment groups. Twenty-seven vitamin D sufficient (25(OH)D >50nmol/l) postmenopausal women were recruited as a concurrent control group. Treatment participants attended four study visits (after screening) at baseline and at 5 (+/-2), 28 (+/-3), and 84 (+/-5) days after administration of the vitamin D bolus. A comprehensive range of biochemical and functional measurements were carried out to give a holistic assessment. Other biochemical measurements included, free 25(OH)D (directly measured and calculated), parathyroid hormone, FGF-23, serum calcium, ionized calcium, urinary calcium excretion and bone turnover markers. Grip strength and a Short Physical Performance Battery were used to assess muscle strength and function. Cardiovascular outcomes were also assessed, including postural changes in blood pressure and the aldosterone-renin ratio.

Despite a large dose-response effect of bolus dose supplementation on total 25(OH)D (& 25(OH)D₃) and total 1, 25(OH)₂D, there was no evidence of a disproportionate rise in free metabolites. The proportional increases in free 25(OH)D (either calculated or directly measured) were in line with the proportional increases in total 25(OH)D (and 25(OH)D₃) across all study time points in all treatment groups. Similarly, the proportional increases in free 1, 25(OH)₂D are in line with the proportional increases in total 1,25(OH)₂D across all time points in all treatment groups. The percentage free 25(OH)D (derived from the ratios of calculated free 25(OH)D to total 25(OH)D and calculated free 25(OH)D₃ to total 25(OH)D₃) were not different between treatment groups at any time point and did not change across the study period within any treatment group. The percentage free that was derived from the ratio of measured free 25(OH)D to total 25(OH)D show a slight increase from baseline at week 1 in the 500 000IU treatment group that was significantly different to the other treatment groups. However, the percent free remained in the normal range and was comparable with percentages reported in healthy adults (0.02-0.09%). The percent free 1, 25(OH)₂D did not change from baseline in any treatment group and did not differ between treatment groups at any time point. The percent free 1, 25(OH)₂D in all treatment groups and at all time points was also in line with the 0.4% reported by other authors in healthy participants.

Taken together, the data presented indicates that there is little evidence to support the hypothesis of a disproportionate rise in free 25(OH)D or free 1, 25(OH)₂D after a single large bolus dose in this vitamin D deficient, but otherwise healthy, older female population. It is therefore unlikely that the adverse events that have been reported after a single large bolus dose previously are caused by excess or disproportionate levels of free vitamin D metabolites.

Despite the large increases in vitamin D metabolites, there was no evidence of hypercalcemia in any treatment group. There was evidence of a fall in PTH in all treatment groups (although there was no dose-response effect) and there was some evidence of increases in FGF-23 and increases in urinary calcium excretion after bolus dosing. The increase in FGF-23 and urinary calcium excretion demonstrated in two of the treatment groups, coupled with the fall in PTH observed at different time points across all treatment groups, suggests that the catabolic pathways for vitamin D metabolites respond rapidly to the sharp increases in vitamin D metabolites in circulation after a large bolus dose. Taken together this again reinforces the theory that the homeostatic mechanisms for the vitamin D and calcium are robust and effectively prevent hypercalcemia after a single large oral dose of vitamin D₃ in healthy older adults.

There was also no effect of treatment on cardiovascular outcomes, including blood pressure and ARR. It has been hypothesised that a single large bolus dose of vitamin D may lead to postural hypotension and that this may be the mechanism of the increased rate of falls reported in some studies. However, our data do not support this

hypothesis.

There were no adverse effects of treatment on physical function, assessed by SPPB and grip strength. We did not see a benefit of supplementation in the largest bolus dose groups, but in the context of this study, it is important to note that we did not see any adverse effects on physical function measures in these treatment groups. As previously described, some studies have demonstrated a link between single large bolus doses of vitamin D and large repeated dose of vitamin D₃ and an increase in falls and fracture. Our data would not support a decline in physical function as the explanation for these findings.

Interestingly, there was evidence of a transient increase in bone turnover markers 1-week after administration in the 500 000IU treatment group. Osteocalcin increased by approximately 23%, PINP by 9% and CTX by 26% by 1-week after administration. Osteocalcin and CTX had fallen to baseline levels by week 4, but PINP remained elevated at week 4 by approximately 15%, before falling to baseline levels by week 12. The transient increase in CTX, OC and PINP was not observed with the lower doses. This is in line with previous findings that have also demonstrated a transient increase in bone resorption markers after a single large bolus dose. Higher levels of bone turnover are associated with bone loss and some studies have shown that higher markers of bone turnover are associated with a greater risk of fracture. It is not clear if these transient changes in increases in BTMs immediately after bolus dosing are clinically relevant, but this mechanism may help to explain the increase in fractures immediately after administration that has previously been reported and requires further investigation.

This is the first study to try and elucidate the mechanism of why people might fall and fracture more after a single large bolus dose of vitamin D₃. This is the first study to show that a single oral bolus dose of up to 500 000IU does not appear to cause a disproportionate rise in free vitamin D metabolites compared to total metabolites. Total 25(OH)D has been measured using more than one method, including measurement by the gold standard LC-MS/MS method. This is the first study to report total 25(OH)D₃ measured by LC-MS/MS after a 500 000IU bolus dose of vitamin D₃. A variety of methods have been used to measure and calculate free 25(OH)D in this study to give a comprehensive assessment of this metabolite. This is also the first study to report the effects of a single large D₃ bolus on the ARR and the first study to report the effects of a vitamin D bolus on postural changes in blood pressure.

To conclude, a single large bolus is up to 500 000IU appears to be well tolerated in healthy older adults. There was little evidence of a disproportionate rise in free vitamin D metabolites after a single large bolus dose (up to 500 000IU). Our data also does not suggest that hypercalcemia, poorer physical function and postural hypotension explain the increase in falls that had been previously reported after a large bolus. The exact mechanism of falls

remains unclear. A transient increase in BTMs 1-week after a 500 000IU bolus dose may explain the increase in fractures immediately after large bolus dose administration that has previously been reported and requires further investigation.

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Contributions

Dr Walsh and Professor Eastell wrote the original grant for funding from the Royal Osteoporosis Society. Dr Walsh was responsible for all clinical aspects of the study.

I was responsible for the development of the protocol. I completed the research ethics application and successfully defended the study, with Dr Walsh, at a Health Research Authority research ethics committee meeting. I was responsible for the setup and maintenance of the site file, submission of protocol amendments and general study management. I also wrote the study documentation, including the participant information sheet, consent form, recruitment adverts and case report forms. I was responsible for study recruitment; I sent emails to hospital trust and University of Sheffield staff, identified GP practices willing to perform mail-outs and put-up poster adverts. I was also responsible for the telephone screening of all volunteers and I wrote the study invitation and appointment letters. I prepared and posted the participant documentation packs.

I booked and co-ordinated all participant visits, including arranging participant travel where necessary and participant expenses. I took consent from all participants and performed all anthropometry and physical function assessments. Phlebotomy and clinical assessments (e.g. blood pressure) at each study visit were carried out by the research nurses at the Clinical Research Facility (Northern General Hospital). Administration of the vitamin D bolus was also carried out by a research nurse. Prescription of the vitamin D was done by Dr Walsh. I completed the data collection forms for each study visit and was responsible for managing participant folders. I completed all data entry and quantitatively coded the sunlight exposure questionnaires. I did all diet diary data entry and analysis. Data checking was carried out by Jill Thompson.

Fatma Gossiel performed most of the biochemical analysis at the Bone Biochemistry Laboratory (University of Sheffield). I performed some of the manual assays and automated analyses under the guidance of Fatma Gossiel. The vitamin D screening and some other biochemical tests were carried out by the Clinical Chemistry and Immunology Departments (Sheffield Teaching Hospitals, Sheffield, UK). Total 25(OH)D₂ and 25(OH)D₃ were measured by Professor Brian Keevil and his team at the laboratory of the Institute of Human Development (University of Manchester, UK). Statistical support and determination of the power calculation was provided by Dr Richard Jacques (School of Health and Related Research (SchARR), University of Sheffield, UK).

Dr Walsh, Professor Eastell and Professor Hill provided guidance and direction throughout this work.

List of abbreviations

1, 25(OH) ₂ D	1, 25-dihydroxylatedvitaminD
25(OH)D	25-hydroxylatedcholecalciferol (25-hydroxylatedvitaminD)
ARR	Aldosterone-Renin Ratio
AUC	Area Under the Curve
BALP	Bone Alkaline Phosphatase
BMI	Body Mass Index
BMU	Bone Multicellular Unit
CTX	Carboxy-terminal collagen crosslinks
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
FGF-23	Fibroblast Growth Factor-23
FGFR	Fibroblast Growth Factor Receptor
iCa	Ionised Calcium
IQR	Interquartile Range
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LRP5	Lipoprotein Receptor-related Protein 5
M-CSF	Macrophage-Colony Stimulating Factor
NCX1	Sodium/Calcium Exchanger
NPT2a/ NPT2c	Sodium-phosphate Cotransporters
NDNS	National Diet and Nutrition Survey
OC	Osteocalcin
PINP	Procollagen 1 N-terminal propeptide
PMCA1b	Plasma Membrane Calcium Pump 1b
PTH	Parathyroid Hormone
RANK	Receptor Activator Nuclear Factor-kB
RANKL	Receptor Activator Nuclear Factor-kB Ligand
RAS	Renin-Angiotensin System
RXR	Retinoid X Receptor
sCa	Serum Calcium
sCr	Serum Creatinine

sPhos	Serum Phosphate
SD	Standard Deviation
SZA	Solar Zenith Angle
TRPV6	Transient Potential Vanilloid Type 6uR
UrCa	Urinary Calcium
urCa:urCr	Urinary Calcium to Creatinine Ratio
VDREs	Vitamin D Response Elements
VDR	Vitamin D Receptor
VDBP	Vitamin D Binding Protein
ARR	Aldosterone-Renin-Ratio

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List of abstracts and publications & declaration

Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously been presented for an award at this, or any other, university.

Oral presentations

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* Serum 25(OH)D deficiency in young adults is associated with low dietary vitamin D intake and a shorter 25(OH)D₃ half-life. *Association for Nutrition Yorkshire & Humber Regional Conference*, 24th October 2018; York University, UK.

Bowles, S. D., Walsh, J.S., Eastell, R., & Hill, T. Change in free 25(OH)D after high dose in vitamin D deficient postmenopausal women. *Human Nutrition Research Centre Research Day*, 26th October 2016; Newcastle University, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* Young adults are at risk of vitamin D deficiency due to low dietary intake and a shorter 25(OH)D₃ half-life. *Rank Prize Funds Mini-Symposium on Nutrition*, 5th October 2016; Grasmere, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* Serum 25(OH)D deficiency in young adults is associated with low dietary vitamin D intake and a shorter 25(OH)D₃ half-life. *The 4th Joint Meeting of European Calcified Tissue Society (ECTS)*, 25 - 28 April 2015; Rotterdam, The Netherlands.

Bowles, S. D., Evans, A.L., Jacques, R.M., *et al.* Serum 25(OH)D deficiency in young adults is associated with low dietary vitamin D intake and a shorter 25(OH)D₃ half-life. *Human Nutrition Research Unit (HNRC) 20th Anniversary Symposium*, 15th October 2014; Newcastle University, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* The effect of age on vitamin D metabolism. *Newcastle University SAFRD Conference*, 21st May 2014; Newcastle University, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* Serum 25(OH)D deficiency in young adults is associated with low dietary vitamin D intake and a shorter 25(OH)D₃ half-life. *5th Mellanby Centre Research Day*, 5th December 2014; University of Sheffield, UK.

Poster presentations

Bowles, S. D., Walsh, J.S., Eastell, R., & Hill, T. Effect of high dose vitamin D on free 25(OH)D and ionised calcium in vitamin D-deficient postmenopausal women. *American Society for Bone Mineral Research Annual Meeting*, 28th September - 1st October 2018; Montreal, Canada.

Bowles, S. D., Walsh, J.S., Eastell, R., & Hill, T. Change in free 25(OH)D after high dose in vitamin D deficient postmenopausal women. *Mellanby Centre Research Day*, 2017. University of Sheffield, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., *et al.* Serum 25(OH)D deficiency in young adults is associated with low dietary vitamin D intake and a shorter 25(OH)D₃ half-life. *The 4th Joint Meeting of European Calcified Tissue Society (ECTS)*, 25 - 28 April 2015; Rotterdam, The Netherlands.

Bowles, S.D., Walsh, J.S., Eastell, R., & Hill, T. Relative change in free D after high dose in postmenopausal women. *CIMA Research Meeting*, 2016; Liverpool, UK.

Bowles, S.D., Walsh, J.S., Eastell, R., & Hill, T. The effect of different vitamin D bolus doses on the ratio of free 25(OH)D to total 25(OH)D in vitamin D deficient post-menopausal women. *CIMA-CIMAR Meeting*, 2015; Nottingham, UK.

Bowles, S. D., Evans, A.L., & Jacques, R. M., et al. Young adults are at risk of vitamin D deficiency due to low dietary intake and a shorter 25(OH)D₃ half-life. *CIMA Physical Activity Meeting*, May 2014.

Publications

Schwartz, J. B., Gallagher, C., Jorde, R., Berg, V., Walsh, J., Eastell, R., **Bowles, S, D.** . . . Bikle, D. (2018). Determination of free 25(OH)D concentrations and their relationships to total 25(OH)D in multiple clinical populations. *The Journal of Clinical Endocrinology and Metabolism*. doi:10.1210/jc.2018-00295

Walsh, J.S., Evans, A.L., **Bowles, S.**, Naylor, K. E., Jones, K.S., Schoenmakers, I., Jacques, R.M., Eastell, R. (2016). Free 25-hydroxyvitamin D is low in obesity, but there are no adverse consequences for bone health. *The American Journal of Clinical Nutrition*, 103:1465–71.

Walsh, J. S., **Bowles, S.**, & Evans, A.L. (2017). Vitamin D in Obesity. *Current Opinions in Endocrinology Diabetes & Obesity*, 24: 389-394.

Chapter 1:

Introduction

1.1 Introduction to vitamin D

Vitamins are organic compounds that are essential for metabolic processes in the human body. They cannot be synthesized endogenously and therefore need to be obtained through the diet (1). Therefore, vitamin D is technically not a vitamin as it is not an essential dietary component, but is a prohormone (a precursor to an active hormone) that can be synthesized in the skin (2).

There are two main types of vitamin D: vitamin D₃ (cholecalciferol) and vitamin D₂ (ergocalciferol) (**figure 1**). Vitamin D₂ is produced from the UVB irradiation of ergosterol (a steroid found in the cell membrane of fungi) and so small amounts of vitamin D₂ are present in plants contaminated with fungi (3). Vitamin D₃ is the animal form and the only form that can be synthesized in the skin upon exposure to UVB radiation (2).

Low levels of vitamin D are common in the UK across a wide range of population sub-groups (3). Vitamin D is essential for the regulation of calcium homeostasis and a casual role in the maintenance of bone health is well established. The Vitamin D receptor (VDR) and vitamin D metabolising enzymes are distributed widely in tissues throughout the body, indicating a diverse range of actions for the vitamin D endocrine system (4). Many observational studies link low vitamin D levels to extra-skeletal diseases such as, cardiovascular disease, cancer, atopy and asthma, neurologic disorders and overall mortality (4, 5). However, intervention studies for extra-skeletal health effects are so far inconclusive.

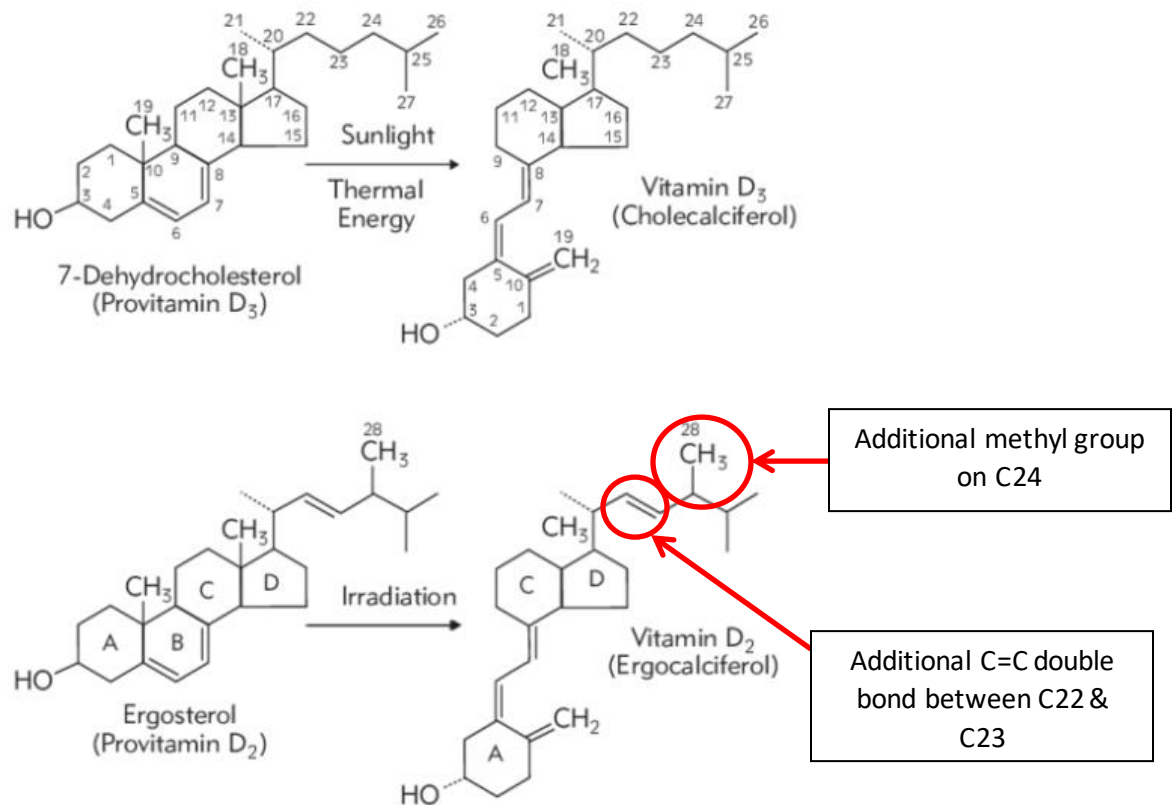
There are still uncertainties over how to best correct vitamin D deficiency. Compliance with daily supplementation is usually problematic in some population sub-groups (6, 7) and so larger bolus doses have been advocated. However, there are still uncertainties around the frequency of dosing and the optimum bolus to use. Adverse consequences have also been reported after a large bolus dose or high dose intermittent vitamin D in some studies, including an increase in falls and fracture (8-10). The mechanisms of these adverse events are still a topic of debate.

1.2 Molecular overview of vitamin D

The molecular structure of Vitamin D₂ and D₃ are closely aligned to that of steroid hormones (such as aldosterone, estradiol and cortisol) because they contain the same cyclopentanoperhydrophenanthrene (a carbon skeleton of three cyclohexane rings and a cyclopentane ring) ring structure (2). Vitamin D is actually a secosteroid because of the broken 9, 10 Carbon-Carbon bond in the B-ring of its cyclopentanoperhydrophenanthrene structure (**figure 1**).

Vitamin D₂ is different structurally to vitamin D₃ in the side chain attached to the secosteroid skeleton, which contains a double bond between carbon atoms 22 and 23 and an additional methyl group (CH₃) on carbon atom 24 (2). As a result of the side chain differences, the molecular mass of vitamin D₃ (384.64 g/mol) is ~3.1% lower than that of vitamin D₂ (396.65 g/mol) (3).

Figure 1: The molecular structures of provitamin D₃, provitamin D₂, vitamin D₃ and vitamin D₂



Source: Adapted from Huotari et al., (1)

1.3 Sources of vitamin D

Vitamin D₃ is obtained by cutaneous synthesis and from foods or dietary supplements. Vitamin D₂ is only obtained from plant based foods and some dietary supplements (3). Cutaneous synthesis of vitamin D₃ is quantitatively the most important source of vitamin D if the skin is regularly exposed to sunlight (11, 12). However, dietary sources of vitamin D become more important if cutaneous synthesis becomes insufficient, due to limited sunlight exposure during summer months and insufficient UVB wavelength containing sunlight during winter months (3).

1.3.1 UVB cutaneous synthesis of vitamin D₃

Vitamin D₃ is formed endogenously in the basal and suprabasal layers of skin epithelial cells (1) when the skin is exposed to UVB radiation (13). For natural solar radiation this is UVB wavelengths of ~280-315nm because the stratospheric ozone prevents shorter wavelengths for passing through the atmosphere (13). UVB radiation causes the photoisomerisation of 7-dehydrocholesterol to previtamin D₃ in the plasma membrane of epidermal skin cells (11, 13, 14). These thermodynamically unstable isomers of previtamin D₃ undergo heat isomerisation, through

rapid rearrangement of double bonds, to the more stable vitamin D₃ (13, 14). Around 50% of previtamin D₃ is converted to vitamin D₃ within two hours (14) and a maximum concentration of vitamin D₃ in the skin is reached within a few hours (11). Vitamin D₃ then enters the extracellular fluid space and binds with vitamin D binding protein (VDBP) in circulation (2, 14).

Regulation of cutaneous synthesis of vitamin D₃

Prolonged sunlight exposure does not lead to vitamin D₃ toxicity. This is because the amount of vitamin D₃ produced in the skin is regulated through the photodegradation of previtamin D₃ and vitamin D₃. Prolonged UVB exposure can lead to the further photoisomerisation of previtamin D₃ into two inert isomers; lumisterol and tacysterol, or isomerisation back to 7-dehydrocholesterol (13). Furthermore, cutaneous vitamin D₃, can also continue to absorb UVB photons and undergo photoisomerisation to biologically inert photoproducts (14, 15) (**figure 2**). If previtamin D₃ levels subsequently fall, these photoconversions are reversible (11). With any prolonged sunlight exposure, a quasi-equilibrium of isomers will result (13) and the amount of previtamin D₃ that is formed is only approximately 12-15% of the original 7-dehydrocholesterol concentration (16).

Figure 2: *The production and regulation of vitamin D₃ in the skin.*

Image removed for copyright reasons. Copyright holder is Elsevier Inc.

Source: *Feldman et al., (14)*

Factors affecting cutaneous synthesis of vitamin D₃

The quantity of vitamin D₃ produced in the skin is dependent upon the amount of exposure to UVB radiation and the efficiency of the production (13). There are several factors that influence the availability of UVB radiation for cutaneous synthesis (13, 17):

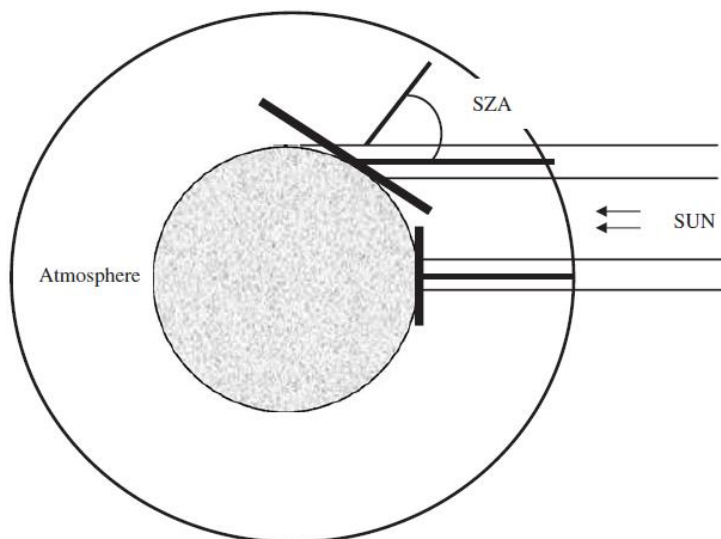
- **Latitude** - UVB radiation is sufficient at the surface for vitamin D production all year round at latitudes below ~37°N (3). Vitamin D is not synthesised at higher latitudes during the winter months (3). Even in the UK, it has been reported that serum total 25(OH)D levels in postmenopausal women in Aberdeen (57°N) were ~10nmol/l higher than in Guildford (51°N), although it is possible that not all of this variation may be due to UVB radiation exposure (18, 19).
- **Season** - The seasonal cycle in vitamin D status has been well observed in regions at mid-to-high latitude (19-22). During the winter period, the amount of UVB radiation in sunlight that reaches the surface is not sufficient for cutaneous synthesis of vitamin D to occur in any meaningful quantity (16).
- **Time of day** - The Solar zenith angle (SZA) is the angle between the local vertical and the position of the sun in the sky (16). The SZA is determined by the latitude, season and time of day. A smaller SZA is associated with lower latitudes, summer months and midday. Conversely, large SZAs are associated with higher latitudes, winter months and early or late in the day (16). The sun is highest in the sky at midday giving more opportunity for cutaneous vitamin D₃ production (**figure 3**).
- **Cloud cover** - Cloud cover can both attenuate and enhance UVB radiation and thus influence vitamin D₃ cutaneous synthesis (23). Fair weather clouds (e.g. cumulus) that do not significantly block sunlight may have little effect on UVB radiation reaching the surface and in some cases may enhance the amount of UVB due to reflectivity (24, 25). UVB radiation will always be attenuated in overcast conditions (26).
- **Surface reflectivity (albedo)** - The albedo of the surface can also increase the UVB radiation available for cutaneous vitamin D₃ synthesis. Most surfaces have a low albedo (e.g. Vegetation: 5%; Soils/rocks: 10%; dry sand: 20%), but snow reflects up to 95% of UVB radiation (27, 28).
- **Air pollution and altitude** - The lower the altitude the more atmosphere UVB radiation has to travel through giving greater chance for attenuation and dispersion. Less UVB radiation will ultimately reach the surface (13, 29). Pollutants reduce UVB radiation at the surface through attenuation and scattering (13). The effect of pollutants is reduced at higher altitude as pollutants tend to accumulate at lower layers in the atmosphere (13).

- **Clothing practice** - Clothing acts as a physical barrier between UVB radiation and the skin and therefore prevents cutaneous vitamin D₃ synthesis (30).
- **Sunscreen use** - Sunscreen is very effective at blocking UVB radiation (31). Appropriate application of SPF 15 reduces cutaneous vitamin D₃ synthesis by ~99.9% and SPF 8 by 97.5% (12). Despite this, appropriate use of sunscreen is rare (e.g. some areas missed during application and timing of application) and therefore it is debatable what effect normal usage of sunscreen has on vitamin D production and overall vitamin D status (32).

Figure 3: The Solar Zenith Angle

Large Solar Zenith Angles: At a larger SZA (when the sun is lower in the sky) the UVB radiation follows a relatively long path through the atmosphere giving a greater chance of attenuation and dispersion of the UVB radiation energy over a large surface area.

Small Solar Zenith Angles: At smaller SZAs (when the sun is higher in the sky), the UVB radiation follows a relatively shorter path through the atmosphere giving less chance of attenuation and less dispersion of the radiation energy over a small surface area.



Source: Adapted from Webb et al., (13)

There are also several factors that affect the efficiency of subcutaneous synthesis:

- **Skin pigmentation** - Melanin is a pigment that gives skin a darker colour and protects from sunburn but it also absorbs UVB radiation (33). Skin pigmentation therefore reduces cutaneous synthesis of vitamin D from sunlight exposure as it absorbs a proportion of the UVB radiation that would otherwise be absorbed by 7-dehydrocholesterol. If exposed to the same absolute dose of UVB radiation, a person with darker skin will synthesise less vitamin D₃ than a person with lighter skin.

- **Age** - The amount of 7-dehydrocholesterol in the skin decreases with age and therefore the efficiency of vitamin D₃ synthesis may decrease (34).

1.3.2 Dietary sources of vitamin D₂ and D₃

Vitamin D₃

There are limited dietary sources of vitamin D in the UK. Naturally rich food sources are of animal origin and contain vitamin D₃. Oily fish, such as kipper, mackerel, herring, salmon and sardines, are the most abundant source of vitamin D₃ (5-16µg per 100g)(35). However, there is wide variation in the vitamin D₃ content in wild and farmed fish and also in the same wild species caught in different geographical locations (36). Egg yolk contains a significant amount of vitamin D₃ (12.6µg per 100g). Other animal products such as meat, fat, kidney and liver also contain vitamin D₃ (0.1-1.5µg per 100g)(35), as well as the 25(OH)D₃ metabolite which is approximately 5 times more potent at increasing vitamin D status than vitamin D₃ (37, 38).

Vitamin D₂

Food sources of vitamin D₂ are extremely limited. Wild mushrooms are a rich natural source of vitamin D₂ (13-30µg per 100) (39). However, cultivated mushrooms that are typically grown in the absence of light do not contain high amounts of vitamin D₂ (39, 40). There are some commercially available UVB treated mushrooms that are enhanced with vitamin D₂.

Fortification and supplementation

Foods can be fortified with either vitamin D₂ or D₃ that has been commercially produced. Vitamin D₂ is synthesised by UVB irradiation of ergosterol found in fungi and vitamin D₃ is synthesised by the UVB irradiation of 7-dehydrocholesterol found in lanolin (sheep wool). In the UK, food products, such as breakfast cereals and margarines, are fortified on a voluntary basis (the mandatory requirement for margarine fortification in the UK was removed in 2013)(3). The European Union law (Directive 2006/141/EC) states that infant formula (1-2.5µg per 100kcal) and follow-on formula (1-3µg per 100kcal) must be fortified with vitamin D. Vitamin D supplements can contain vitamin D₂ and D₃, but in the UK largely contain vitamin D₃.

1.4 Vitamin D metabolism

1.4.1 Absorption of dietary vitamin D

Vitamin D is lipid soluble and absorbed at the small intestine. Vitamin D is likely associated with mixed micelles and is likely taken up by passive diffusion into the enterocytes (41, 42). Within the enterocytes, vitamin D is packaged into chylomicrons before passing into the lymphatic system and then into systemic circulation (41, 43). Therefore, vitamin D may require the presence of other lipids in the duodenum to stimulate bile acid secretion for the facilitation of lipid absorption at the intestinal mucosa (42). A systematic review of the effect of different vehicles on the absorption of vitamin D has reported that a lipid based vehicle (oil) is optimal compared to powders and ethanol (44), but absorption does still occur without fat or oily vehicles (42).

1.4.2 Transport of vitamin D in circulation

Vitamin D₃ produced cutaneously enters the extracellular fluid and diffuses into dermal capillaries (45). In circulation, it is bound to VDBP and transported to the liver (46). Vitamin D has a relatively low binding affinity for VDBP with estimates between 1×10^{-5} and 1×10^{-7} mol/L (47).

Dietary vitamin D₂ and D₃ is transported to the liver in chylomicrons via the lymphatic system and then circulation (46). Vitamin D has a relatively short plasma half-life of 4-6 hours (48). This is, in addition to its low affinity for VDBP, a consequence of chylomicron transport because vitamin D is taken up by peripheral tissues, such as adipose and muscle, due to the action of lipoprotein lipase (49). Any remaining vitamin D in the chylomicron remnant can be quickly taken up by the liver (49). This loss into tissue and liver pools contributes to the relatively short half-life (47).

1.4.3 Hepatic vitamin D hydroxylation to 25(OH)D

Vitamin D₃ and vitamin D₂ are biologically inert (50) and need to be converted to the active metabolite to exert a physiological effect. This occurs in two sequential hydroxylation steps. In the initial step, vitamin D is hydroxylated (the addition of a hydroxyl [-OH] group) in the liver at the 25-carbon position to form 25-hydroxylatedcholecalciferol (25(OH)D) (2, 47, 50-52). This step is mediated by microsomal (CYP2R1) or the mitochondrial (CYP27A1) cytochrome P450 25-hydroxylase enzymes (47, 52). The production of 25(OH)D is substrate dependent as there appears to be no robust regulatory factor on the rate of production (52). The 25(OH)D is then transported from the liver to the kidney via circulation, predominantly bound to VDBP. 25(OH)D has a binding affinity for VDBP (5×10^{-8} mol/l) that is at least an order of magnitude higher than other metabolites (47). This gives 25(OH)D a relatively long circulating half-life of approximately 13-18 days (20, 47, 53) and so total 25(OH)D is the main circulating form measured to assess vitamin D status in humans (52).

1.4.4 Renal hydroxylation of 25(OH)D to 1, 25(OH)₂D

Following hepatic hydroxylation, 25(OH)D is further hydroxylated to 1, 25(OH)₂D; the hormonally active form (2, 47, 52, 54). This step is catalyzed by the mitochondrial P450 1- α -hydroxylase enzyme (CYP27B1), in the proximal tubules of the kidney and in extra-renal tissues (54). Renal production is the main source of 1, 25(OH)₂D in circulation and is the mediator of the endocrine functions of vitamin D. However, CYP27B1 mRNA, protein and enzyme activity have been demonstrated in a wide range of other tissues (2, 55), but the effects of extra-renal 1, 25(OH)₂D seem to be restricted to paracrine and autocrine functions and does not significantly contribute to circulating levels of 1, 25(OH)₂D (2). Following its release into circulation, 1, 25(OH)₂D is predominantly bound to VDBP with an affinity of 2×10^{-7} mol/l; an order of magnitude less than its precursor (47). The active metabolite has a relatively short half-life of 10-20 hours (47).

1.4.5 Regulation of 1, 25(OH)₂D production

The renal synthesis of 1, 25(OH)₂D is tightly regulated. The production of 1, 25(OH)₂D is upregulated by parathyroid hormone (PTH) as a signal of calcium homeostasis and downregulated by Fibroblast Growth Factor-23 (FGF-23) as a signal of phosphate homeostasis (52).

PTH plays an important role in regulating calcium homeostasis. The calcium sensing receptors in the parathyroid gland stimulate the secretion of PTH in response to a fall in circulating ionised calcium levels (50). PTH stimulates the synthesis of CYP27B1 (56) in the proximal cells of the kidney and this increases renal 1, 25(OH)₂D production (52, 57). To increase circulating calcium levels, 1, 25(OH)₂D increases calcium absorption at the intestine, reduces calcium excretion at the kidney and stimulates the resorption of calcium from bone (2, 50, 51). There is also a direct negative feedback on 1, 25(OH)₂D production by 1,25(OH)₂D through the downregulation of gene expression for CYP27B1 (52). When circulating levels of 1, 25(OH)₂D are low, then renal production is high and when circulating levels of 1, 25(OH)₂D are high, renal production decreases (2).

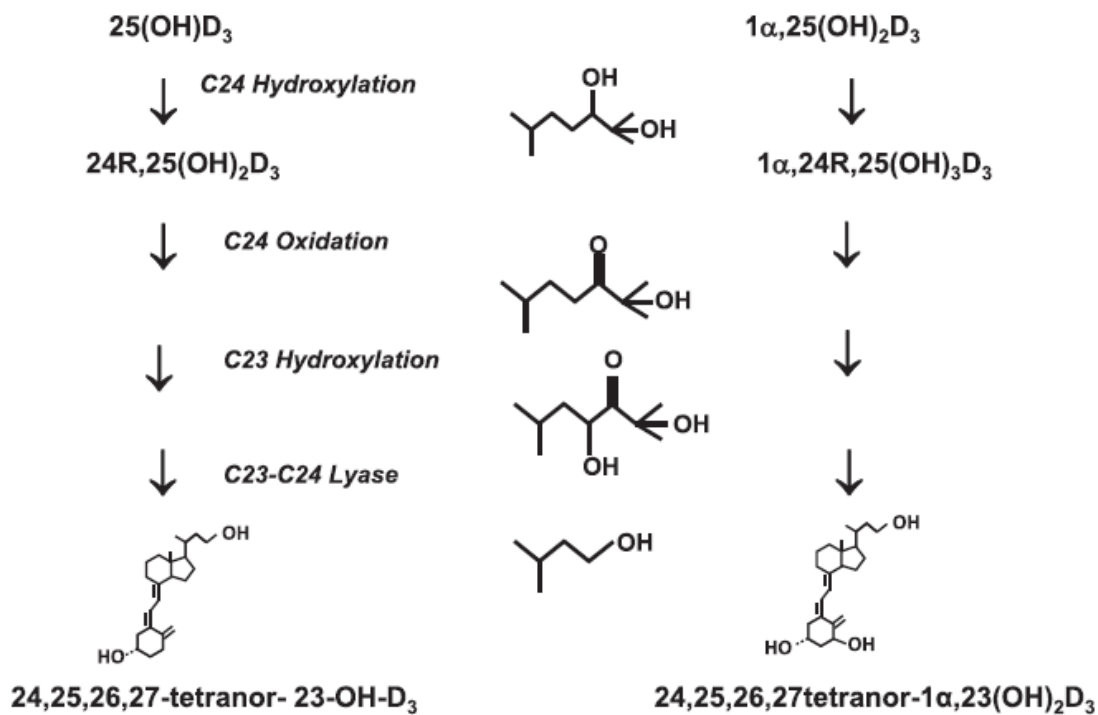
FGF-23 mediates the regulatory effect of circulating phosphate concentrations on lowering circulating 1, 25(OH)₂D (58). As well mediating calcium homeostasis, 1, 25(OH)₂D stimulates the absorption of phosphate at the intestine and the release of phosphate from bone (52). FGF-23 is secreted by osteoblasts and osteocytes in response to elevated circulating phosphate concentrations (52). It appears to downregulate the synthesis of 1, 25(OH)₂D by inhibiting the transcription of CYP27B1 at the kidney (59) to modulate circulating phosphate concentrations.

1.4.6 Catabolism and excretion of vitamin D metabolites

The degradation of 25(OH)D and 1, 25(OH)₂D is catalysed by CYP24 (a 24-hydroxylase enzyme produced in the kidney). Therefore, the CYP24 mediated catalytic pathway is important in limiting the action of 1, 25(OH)₂D in

target tissues (52). The hydroxylation of carbon-24 is the first in a series of four steps that ultimately produce inactive water-soluble compounds that can then be excreted in bile (**figure 4**). Within a target cell, $1, 25(\text{OH})_2\text{D}$ enhances its own degradation by stimulating the expression and activity of CYP24A1, but PTH has inhibitory effects on CYP24 gene transcription in the kidney (52).

Figure 4: A summary of the reactions catalysed by CYP24A1 to inactivate $25(\text{OH})\text{D}$ and $1, 25(\text{OH})_2\text{D}$

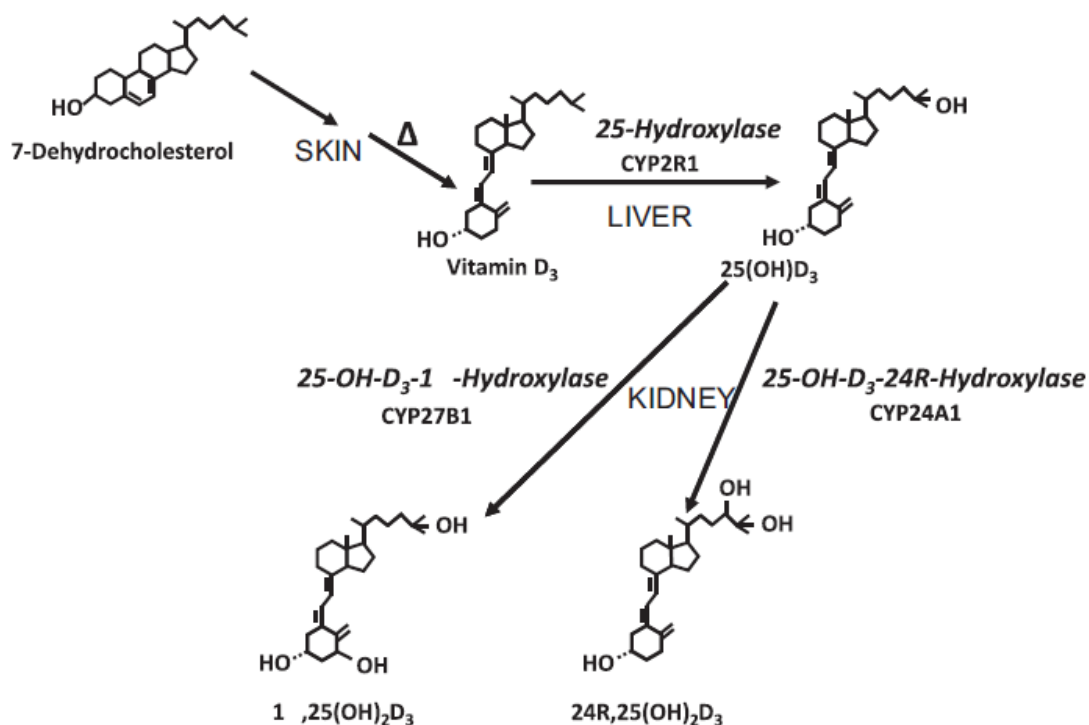


Source: Henry (52)

1.4.7 Summary vitamin D metabolism

Vitamin D_3 is formed by the UVB irradiation of 7-dehydrocholesterol found in the skin. Vitamin D_3 enters circulation and is transferred to the liver (predominantly bound to VDBP). Dietary vitamin D_3 is absorbed at the intestine and transported to the liver (predominantly found in chylomicrons). At the liver, the side chain of Vitamin D_3 is hydroxylated at the 25-carbon position. The resulting $25(\text{OH})\text{D}_3$ is the major circulating form and is used to assess vitamin D status. At the kidney, $25(\text{OH})\text{D}_3$ is further hydroxylated at the 1-carbon position or the 24-carbon position to form either $1, 25(\text{OH})_2\text{D}$ (the active hormone) or $24, 25(\text{OH})_2\text{D}_3$ (the inactive water-soluble compound ready for excretion in bile). Vitamin D_2 that has been absorbed at the intestine from dietary sources undergoes similar metabolic transformations.

Figure 5: A simple overview of vitamin D metabolism



Source: Henry (52)

1.5 Mechanisms of action for 1, 25(OH)₂D

The active vitamin D metabolite, 1, 25(OH)₂D, exerts a biological response through the regulation of gene transcription (genomic responses) or by activating signal transduction pathways within or near the plasma membrane of the cell (rapid or non-genomic responses)(60, 61). The mechanism of action is modulated through the binding of 1, 25(OH)₂D with a vitamin D-receptor (VDR) (2, 50, 60, 61).

The VDR has a very high binding affinity for 1, 25(OH)₂D, but only binds 0.1-0.3% as well with 25(OH)D (2) and the VDR does not bind with vitamin D₃ or Vitamin D₂ (2). There does not appear to be any difference in binding affinity between 1, 25(OH)₂D₃ and 1, 25(OH)₂D₂ (62).

The VDR is expressed in cells that are integral to calcium and phosphate homeostasis, but is also found in a wide-range of other tissues (2, 63). **Table 1** gives an overview of VDR expression in different tissues and cells (63).

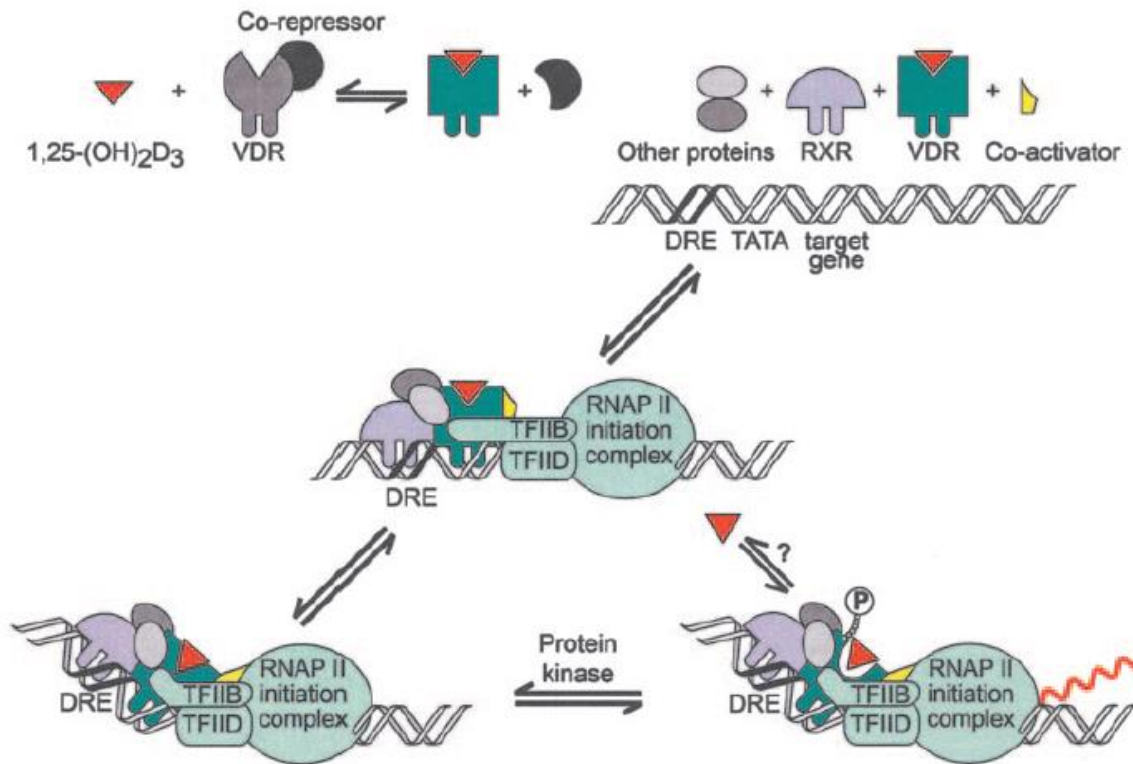
Table 1: A summary of the distribution of VDR expression in tissues/cells (63)

Organ and Tissue	Expression level
<i>Digestive System</i>	
Small intestine	Very strong
Large intestine	Strong
Liver	None
Pancreas	Weak
<i>Kidney</i>	
Distal tubule	Very Strong
Proximal tubule	Weak
Glomerular podocytes	Very weak
<i>Endocrine System</i>	
Thyroid	None
Parathyroid	Very Strong
Pituitary gland	Weak
Adrenal gland	None
<i>Respiratory System</i>	
Lung alveolar cells	None
Bronchus	Moderate
<i>Bone</i>	
Osteoblasts	Moderate
Chondrocytes	Very weak
<i>Muscle</i>	
Cardiac	None
Smooth	None
<i>Immune System</i>	
Thymus	Moderate
Spleen/lymph node	Weak
<i>Reproductive System</i>	
Testis	Weak
Prostate gland	Moderate
Mammary gland	Moderate

1.5.1 Genomic response

Gene expression is regulated by the active vitamin D metabolite. 1, 25(OH)₂D enters the target cell and binds with the VDR in the nucleus. When occupied by 1, 25(OH)₂D, the VDR then forms a heterodimer with the retinoid X receptor (RXR) that binds to vitamin D response elements (VDREs) that are found in the promoter region on vitamin D regulated genes (50, 51, 64). At the same time, the complex binds several other proteins required to complete the transcription complex (e.g. it acquires a co-activator or co-repressor) (50, 64). Once the complex has been formed, the DNA kinks (65) and the VDR becomes phosphorylated (50) or dephosphorylated (64). The result of this regulation is either suppression or activation of transcription (50). A summary of the proposed molecular mechanisms of gene induction and repression by the 1, 25(OH)₂D-VDR complex is shown in **figure 6**.

Figure 6: A schematic representation of the regulation of gene expression by 1, 25(OH)₂D



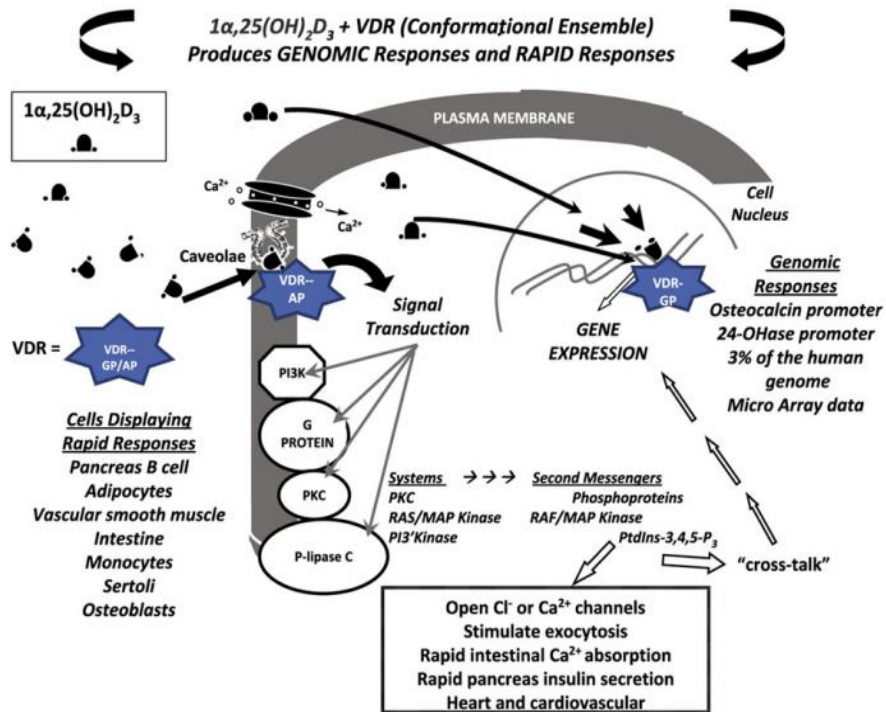
VDR; Vitamin D Receptor, **RXR;** Retinoid X Receptor, **TFIIB;** Transcription Factor IIB, **TFIID;** Transcription Factor IID, **DRE;** Vitamin D Response Element, **RNAP;** RNA polymerase

Source: DeLuca (50)

1.5.2 Non-genomic response (Rapid response)

Vitamin $1, 25(\text{OH})_2\text{D}$ is conformationally flexible and so in addition to mediating genomic responses, it has also been demonstrated to initiate biological responses via rapid response pathways (61). Some biological responses that are known to be mediated by $1, 25(\text{OH})_2\text{D}$ occur too quickly to be explained via the genomic response mechanism, which take several hours or days to take effect and can be blocked or attenuated by inhibitors of transcription and translation (61). Rapid responses typically occur within minutes (61). This rapid response is mediated by the binding of $1, 25(\text{OH})_2\text{D}$ to the VDR found in caveolae (flask-shaped membrane invaginations found in the cell membrane of a variety of cells) (2, 66). The binding of the caveolae-associated VDR elicits a cellular response through the activation of one or more second messengers (e.g. Protein kinase C, G-protein coupled receptors, Phospholipase C or Phosphatidylinositol-3-kinase (PI3K), mitogen-activated protein (MAP) kinase or Cyclic Adenosine Monophosphate (cyclic AMP)) (2). There is evidence to show that these rapid response mechanisms appear to operate in the intestine, pancreatic β -cells and osteoclasts (61). A schematic summary of the $1, 25(\text{OH})_2\text{D}$ activation of non-genomic (rapid response) and genomic cellular signaling pathway is shown in **figure 7**.

Figure 7: A schematic summary of the $1, 25(\text{OH})_2\text{D}_3$ activation of non-genomic (rapid response) and genomic cellular signaling pathway



Source: Haussler et al., (61)

1.6 Physiological roles of vitamin D

1.6.1 Regulation of $1, 25(\text{OH})_2\text{D}$ synthesis

An overview of vitamin D metabolism can be found in **figure 8**. The main physiological function of the active vitamin D metabolite is the regulation of calcium and phosphate metabolism which is essential for normal bone mineralisation (62, 64). Calcium homeostasis is also essential for neuromuscular function (50). Circulating total calcium concentrations are tightly regulated at 2.10 - 2.50 mmol/l, and ionized levels (free calcium) makes up approximately 50% of this value (1.15 - 1.30 mmol/l). This level of calcium is adequate for healthy bone mineralisation (50).

There are calcium sensing transmembrane receptors located in the parathyroid cells of the parathyroid gland that detect circulating calcium concentrations. When even a small decrease in circulating calcium concentration is detected by these transmembrane proteins, they stimulate the secretion of PTH into circulation from the parathyroid gland (50). The compensatory increase in PTH stimulates CYP27B1 in the proximal convoluted tubule

cells for optimal production of 1, 25(OH)₂D (50, 51, 67) and inhibits CYP24A1 (57, 67, 68).

The active vitamin D hormone then seeks to increase circulating calcium levels in three different ways; by the direct stimulation of intestinal calcium absorption, or together with PTH, the reabsorption of calcium at the kidney and the resorption of calcium from bone (50, 67). The resulting increase in circulating calcium concentration is detected by the calcium sensing receptors on the parathyroid glands and acts as a negative feedback on PTH secretion (50). There is also a direct negative feedback mechanism of 25(OH)D (69) and 1, 25(OH)₂D (51) to PTH.

The increase in active vitamin D also simultaneously increases serum phosphate concentrations. FGF-23 is stimulated in response to elevated circulating phosphate concentrations. Klotho, a highly expressed transmembrane protein in the distal tubule of the kidney, forms complexes with FGFRs and acts as a co-receptor for FGF23 (61). FGF-23 and klotho induce the expression of CYP24A1 and suppress the expression of CYP27B1, and therefore promote the catabolism and inhibit the synthesis of 1, 25(OH)₂D (61, 70).

If circulating calcium concentrations become elevated the parafollicular cells (C-cells) of the thyroid gland secrete calcitonin (a peptide), which blocks the mobilisation of calcium and phosphate from bone by inhibiting osteoclast activity and inhibits the renal reabsorption of calcium and phosphate (61).

Figure 8: A summary of vitamin D metabolism

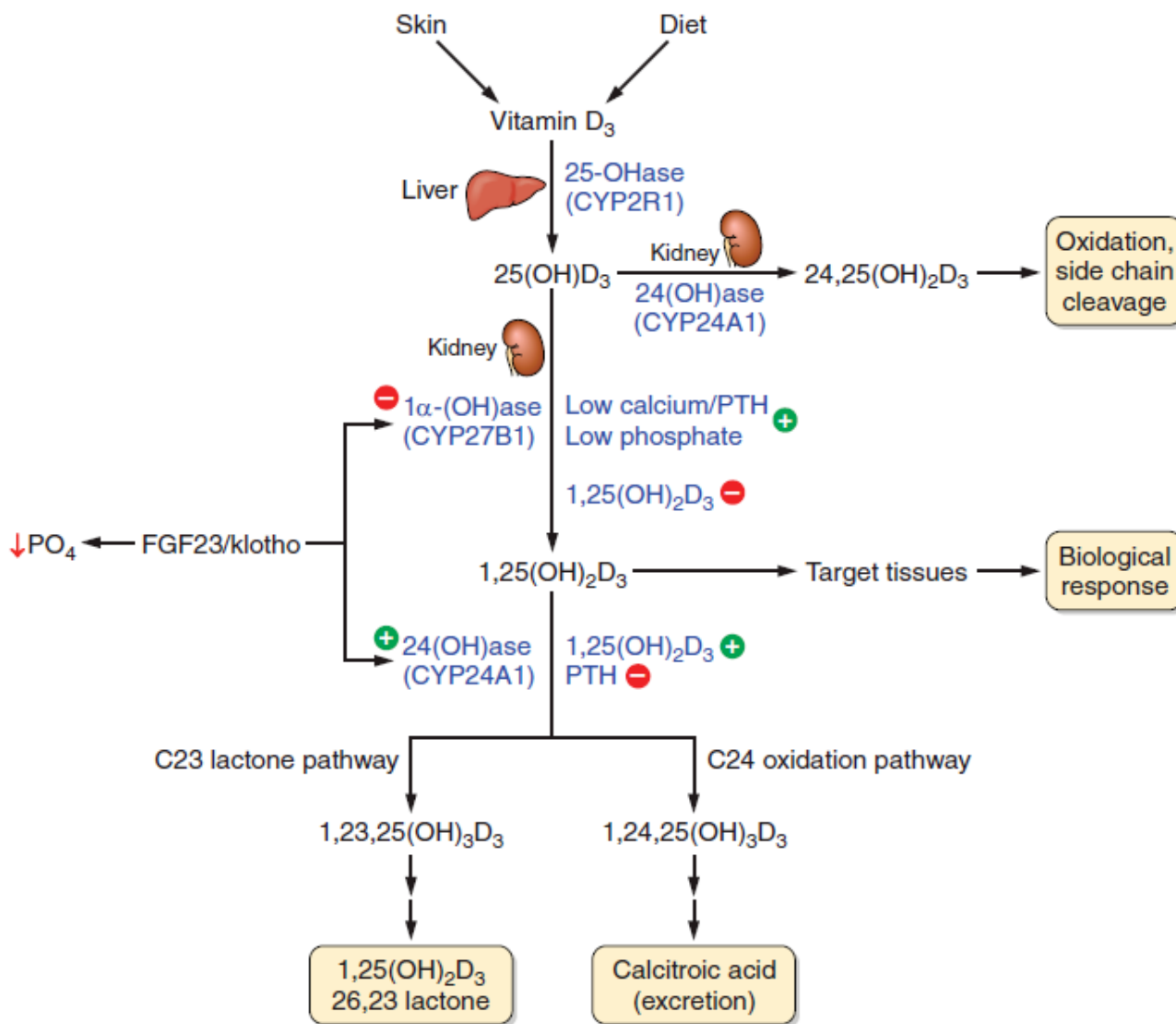


Diagram from: Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: metabolism, molecular mechanism of action, and pleiotropic effects. *Physiological Reviews*, 2016, Page 386.

Source: Christakos et al., (67)

1.6.2 Intestinal calcium absorption

The principal action of 1, 25(OH)₂D is stimulating intestinal calcium absorption (4, 64, 67). Dietary calcium is favored to support circulating calcium concentrations in healthy individuals over mobilisation of calcium from bone or renal reabsorption (50, 64). However, during negative calcium balance (insufficient dietary calcium intake to sustain the needs of the organism) but with normal vitamin D endocrine functioning, a normal circulating calcium concentration will be maintained at the expense of bone health (4, 50, 64).

Transcellular (active) calcium absorption

The facilitated diffusion model is the most widely accepted and studied mechanism for 1, 25(OH)₂D vitamin D-mediated intestinal calcium absorption (67). The transcellular calcium absorption mechanism is stimulated by 1, 25(OH)₂D in three separate ways: 1) increasing the expression of luminal calcium transport channels that are found in the apical membrane (e.g. TRPV6); 2) increasing the expression of the calcium binding proteins (e.g. calbindin-D) and; 3) increasing the expression of the calcium extrusion systems found at the basolateral membrane (e.g. PMCA1b) (67, 71).

Transcellular calcium absorption seems to mostly occur in the proximal parts of the small intestine; the duodenum and jejunum (71, 72), but this contributes only a small proportion of total calcium absorption (8-10%) (73). Indeed, there is some evidence from rat models that transcellular calcium transport also occurs in more distal regions of the intestine as well, such as the ileum, cecum and colon (74-76) and the highest levels of TRPV6 are reported to be in the distal intestine (77).

When dietary calcium intake is low, the fraction of calcium absorbed by the transcellular pathway is relatively high. When dietary calcium intake is high, the transcellular pathway accounts for a lower fraction of the total calcium absorbed (71, 78, 79).

It has been demonstrated that the transcellular transport of calcium is saturated at very low levels of total 25(OH)D (<10-12.5nmol/l) and this very efficient absorption of calcium explains why people do not necessarily develop osteomalacia if there is adequate dietary intake of calcium (and phosphorus) (80, 81). In vitamin D deficiency, 1, 25(OH)₂D levels are maintained (and therefore transcellular intestinal calcium absorption) by a compensatory increase in PTH, until total 25(OH)D falls below 10-12.5nmol/l where there appears to be insufficient substrate to maintain 1, 25(OH)₂D levels (81). In addition, vitamin D supplementation of 60µg/day of vitamin D₃ has little effect on calcium absorption in vitamin D insufficient younger women (aged 25-45 years) (80) and in older vitamin D insufficient women (57-90 years) daily doses of vitamin D up to 120µg/day only increase the total amount of calcium absorbed by up to 6% (82).

Paracellular (passive) calcium absorption

Calcium is also absorbed via a passive process that occurs across the tight junctions of intestinal epithelial cells (67, 71). This paracellular calcium absorption pathway occurs across the length of the intestine (72), especially in

the ileum and colon (75), and is driven by an electrochemical gradient (71). This pathway is non-saturable and was thought to be vitamin D independent (67). However, some studies have shown that some tight junction proteins (e.g. claudins-2 & claudins-12) that are involved in the paracellular transport process may be upregulated by 1, 25(OH)₂D via the VDR (83). The role of 1, 25(OH)₂D in this calcium transport pathway is up for debate and further research is required.

1.6.3 Renal calcium absorption

Most calcium filtered by the glomerulus is reabsorbed at the proximal and distal tubule and only 1-2% of filtered calcium is lost in urine (67). Around 65% of the filtered calcium is reabsorbed in a passive way (following a sodium gradient) and independent of 1, 25(OH)₂D at the proximal tubules (67). However, in the distal tubules, calcium reabsorption is regulated by PTH and 1, 25(OH)₂D via a transcellular mechanism (similar to the transcellular pathway at the intestine)(67). Calcium enters the cytoplasm via TRPV5 at the apical membrane, binds to calbindin-D and is extruded at the plasma membrane by a sodium/calcium exchanger (NCX1) and PMCA1B (67). Phosphate transport at the proximal tubule occurs via sodium-phosphate cotransporters NPT2a and NPT2c. Phosphate reabsorption is regulated by PTH, 1, 25(OH)₂D and FGF-23. The abundance of NPT2a/c is decreased by PTH (84) and FGF-23 (85), which promotes renal loss of phosphate.

1.6.4 Bone resorption

Overview of bone biology

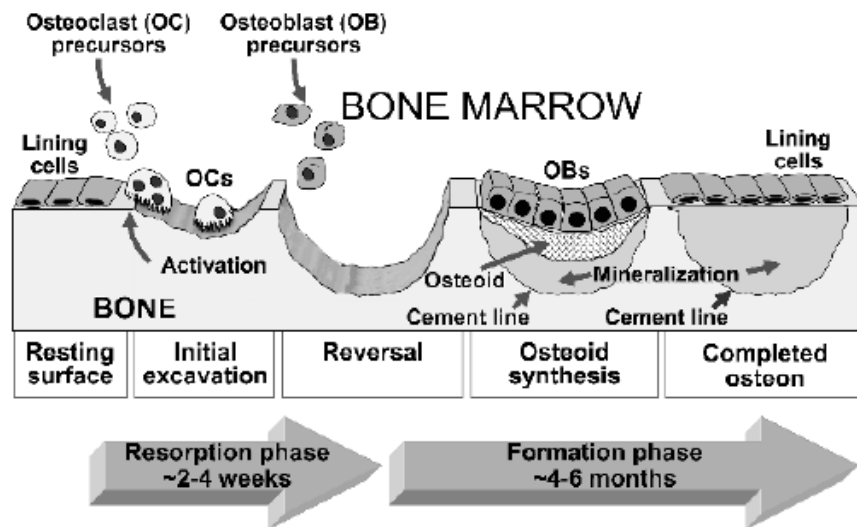
Bone consists of an organic matrix, bone cells and minerals. The inorganic material mainly consists of calcium and phosphate in a crystalline form called hydroxyapatite. The organic material is mainly type I collagen (flexible fibres) that acts as a scaffold or framework for the deposition of calcium hydroxyapatite crystals is a process called mineralisation (86). It is the mineralisation of the collagen scaffold by hydroxyapatite crystals that gives bone its strength. This process is facilitated by specialised bone cells known as osteoblasts that secrete collagen fibres and initiate mineralisation. Osteoblasts differentiate into osteocytes as they become trapped in the bone matrix and play an important role in the day-to-day metabolism of bone (87). Osteocytes secrete lysosomal enzymes (e.g. cathepsin-K) that help to digest the bone matrix during bone remodeling (87).

Overview of bone turnover

Bone is a dynamic organ. It is constantly being remodelled in response to the internal and external environment. Remodeling is physiologically essential for maintenance of bone mass, repair of micro damage to the skeleton and for the regulation of calcium homeostasis (86, 88). Bone remodelling occurs through bone resorption and bone formation. These two processes are temporal (formation follows resorption) and spatial; resorption and formation occur in the same place at discrete foci throughout the skeleton known as bone multicellular units (BMUs)(86, 88).

An overview of the bone remodelling cycle is found in **figure 9**. It is an imbalance between the bone resorption and bone formation processes (excessive bone resorption and/or inadequate bone formation) that can lead to a decreased bone mass, microarchitectural deterioration resulting in skeletal frailty and increased fracture risk (86, 88, 89).

Figure 9: A summary of the stages of bone remodelling.



Source: Riggs et al., (88)

- 1. Activation:** The bone lining cells become activated and retract, attracting osteoclasts to the active site (86, 88). Osteoclasts are recruited from precursors in circulation or in the bone marrow.
- 2. Bone resorption:** osteoclasts form a sealing zone with the bone surface and a highly acidic microenvironment allows for optimum catalytic activity of bone resorbing enzymes such as cathepsin K (87).
- 3. Reversal:** Osteoclasts disassociate from the bone surface and are removed by apoptosis and osteoblasts are recruited to the active site from osteoblast precursors (88).
- 4. Bone Formation:** The resorption pit is lined with osteoblasts that secrete osteoid (new) bone that over time becomes mineralized (88). Some osteoblasts become trapped in the matrix and differentiate into osteocytes (86).
- 5. Quiescence:** Some osteoblasts flatten and become new bone lining cells that cover the bone once more and the remodelling process is complete.

The role of 1, 25(OH)₂D in regulation of bone turnover

A number of genes are regulated by the action of 1, 25(OH)₂D in bone cells (64). Osteocytes and osteoblasts respond to 1, 25(OH)₂D by expressing and releasing FGF-23 to control circulating phosphate concentrations which inhibits CYP27B1 and stimulates CYP24A1; all\ to negatively regulate 1, 25(OH)₂D concentrations (57, 64, 68).

Catabolic actions

The primary effect of $1, 25(\text{OH})_2\text{D}$ on bone is to promote bone resorption (64). To increase circulating calcium concentrations, an elevated $1, 25(\text{OH})_2\text{D}$ concentration stimulates osteoblasts to produce receptor activator nuclear factor- κB ligand (RANKL)(50, 90). Osteoclast precursors express the receptor activator nuclear factor- κB (RANK) which recognizes RANKL (91). RANKL stimulates the differentiation of osteoclast precursors to functional osteoclasts to stimulate bone resorption in the presence of osteoblast derived macrophage-colony stimulating factor (M-CSF) (91). Osteoprotegerin, a decoy receptor for RANKL and an inhibitor of osteoclastogenesis, is also repressed by $1, 25(\text{OH})_2\text{D}$ in osteoblasts (90).

Anabolic actions

There are also anabolic actions of $1, 25(\text{OH})_2\text{D}$ and so the active metabolite plays an important role in normal mineralisation of bone. Osteopontin expression (encoded by the SPP1 gene) is enhanced by $1, 25(\text{OH})_2\text{D}$ in osteoblasts and acts to promote osteoblast survival and ossification of bone (61, 64). Osteoblast proliferation is also increased through enhanced Canonical Wnt signaling (92) due to a $1, 25(\text{OH})_2\text{D}$ -mediated upregulation lipoprotein receptor-related protein 5 (LRP5) gene expression (93, 94).

Integrated actions of $1, 25(\text{OH})_2\text{D}$ on bone

To integrate catabolic and anabolic actions of $1, 25(\text{OH})_2\text{D}$, it can be postulated that at lower physiologic levels of $1, 25(\text{OH})_2\text{D}$ (and in the absence of elevated PTH) bone formation is promoted through anabolic actions, but at elevated levels catabolic bone resorption is favoured as $1, 25(\text{OH})_2\text{D}$ acts as an inhibitory signal to mineralisation (64). In support of this, elevated $1, 25(\text{OH})_2\text{D}$ is known to cause impaired mineralisation of bone (95) and *in vitro* (96) and *in vivo* (97) osteoblast-like cell function is suppressed by supraphysiologic levels of $1, 25(\text{OH})_2\text{D}$, but stimulated by physiologic levels. In addition, supraphysiological levels of $1, 25(\text{OH})_2\text{D}$ increased circulating calcium and RANKL expression *in vivo* (91). It is also important to acknowledge that *in vivo*, both $1, 25(\text{OH})_2\text{D}$ and PTH seem to be required for the mobilisation of calcium from bone (91, 98) and so "two keys" are required as a safety mechanism (50).

There is a delicate balance between anabolic and catabolic influence on the skeleton at physiologically optimal levels of $1, 25(\text{OH})_2\text{D}$, with either deficiency or excess being deleterious to bone (64). In deficiency, pathologic bone resorption occurs through PTH signaling of osteoclasts and in excess osteoclastic bone resorption is stimulated through the RANKL signaling pathway (64, 91).

1.6.5 Non-classical physiological functions

Vitamin D also appears to have many extra skeletal effects (4, 5, 99). This conclusion is derived from the observation that the VDR and CYP27B1 are present in a wide number of tissues (63, 100, 101).

The VDR and vitamin D metabolising enzymes are present in many different cells of the innate and adaptive immune systems (102, 103). Observational studies have linked vitamin D deficiency to an increased risk of infection, particularly upper respiratory tract infections (104) and RCTs do provide some evidence for vitamin D supplementation in preventing infections in populations that are vitamin D deficient (105). Vitamin D supplementation may also reduce atopic exacerbations (106), although not all review articles support this conclusion (107).

Vitamin D is also implicated in cell proliferation and cancer development (4). The presence of the VDR has been shown in many cancer cell lines and tissues (108). CYP27B1 is often expressed at higher levels in cancer cells than normal cells. The active vitamin D metabolite also appears to have an anti-proliferative effect on many cancer cells through the inhibition of cell cycle progression (4, 109). Despite this, the existing intervention studies that have so far been conducted do not appear to demonstrate a benefit of vitamin D supplementation on human cancer incidence or progression (4).

The expression of the VDR in muscle is controversial, with some groups unable to detect the protein in rodent and human skeletal muscle (110), but other groups have found the VDR to be widely expressed at the mRNA and protein levels (111, 112). Some studies have found a significant positive association between total 25(OH)D and physical performance (113, 114). The effect of vitamin D supplementation on physical function and falls is controversial, but several meta-analyses have shown a preventative effect of supplementation on falls in older people (115, 116), particularly in those with a baseline total 25(OH)D of <50nmol/l (117, 118).

Observational studies have also demonstrated an inverse relationship between vitamin D status and cardiovascular disease risk (119-123), however, the results from intervention studies are less consistent, with some meta-analyses demonstrating a protective effect of supplementation against cardiovascular risk factors (124) and others demonstrating no protective effect (125-128).

1.7 Assessment of vitamin D status

1.7.1 What metabolite should we measure?

The most commonly used biochemical measurement of vitamin D status is total 25(OH)D. This is because it reflects contributions from cutaneous synthesis and from the diet (129). There is also little or no regulation in the

enzymes that catalyse its synthesis in the liver (52) and because of its relatively high binding affinity to VDBP, 25(OH)D also has a relatively long circulating half-life of 13-18 days (20, 47, 53, 130).

Although 1, 25(OH)₂D is the active hormone and is the driver of physiological responses, it is not a good measure of vitamin D stores in the body. This is because 1, 25(OH)₂D is tightly regulated in circulation by factors other than vitamin D intake and cutaneous synthesis (e.g. serum calcium, PTH, FGF-23) (129, 131). Even when there is vitamin D deficiency, 1, 25(OH)₂D concentrations are normal or even elevated (due to PTH-mediated upregulation of CYP27B1) and total 25(OH)D needs to fall to very low levels (<10nmol/l) for 1, 25(OH)₂D concentrations to significantly fall (81). The active metabolite also has a short half-life of 4-20 hours (47, 132, 133) compared to 25(OH)D due to a weaker binding affinity with VDBP (47). As a result, 1, 25(OH)₂D only circulates in pmol/l concentrations, a thousand-fold less than 25(OH)D.

1.7.2 Definition of vitamin D deficiency, insufficiency and sufficiency

There are several definitions of sufficient circulating total 25(OH)D; most of which are derived from studies of PTH and skeletal responses to circulating total 25(OH)D. There is ongoing debate concerning these thresholds and it is unknown what the optimal total 25(OH)D concentration is for non-skeletal outcomes. In the UK, serum total 25(OH)D <25nmol/l is currently defined by the Scientific Advisory Committee on Nutrition (SACN) as vitamin D deficiency, because this corresponds to the upper level at which osteomalacia or rickets is observed but chronic or less severe effects on bone health may occur below 50nmol/l (134). The threshold is a 'population' threshold, proposing that a total 25(OH)D concentration <25nmol/l is associated with elevated risk of poor musculoskeletal health at the population level (3). The Institute of Medicine (IOM) currently sets a threshold of total 25(OH)D <30nmol/l for deficiency and <50nmol/l for insufficiency (129) However, the Endocrine Society recommends that a total 25(OH)D of >75nmol/l is required for optimum health, with 50-75nmol/l insufficient and <50nmol/l deficient (135). In this thesis, the classifications set by the IOM will be used as these thresholds are endorsed by the Royal Osteoporosis Society and are widely used by UK healthcare practitioners (131).

1.8 Recommendations for dietary vitamin D intake in the UK

In the UK, SACN has recently set a reference nutrient intake (RNI) of 10ug (400IU) per day for individuals aged 4 and over (3). This is the average amount needed to maintain total 25(OH)D above 25nmol/l for 97.5% of the UK population when UVB exposure from the sun is minimal (3). The RNI reflects day-to-day variation in vitamin D intake, is an average intake over a period of time, is applicable throughout the year and refers to intakes from all dietary sources. The RNI is applicable to pregnant and lactating women and other at-risk population sub-groups. No RNI was set for 0-3 year olds but a 'safe intake' was set at 8.5ug/d for 0 - <1 year olds (including exclusive and partially breast fed infants) and 10ug/d for 1 to < 4 year olds (3). Tolerable upper levels were set to match that of

the European Food Safety Authority (EFSA) of 100ug/d (4000IU) for adults and 11-17 years, 50ug/d (2000IU/d) for 1-10 years and 25ug (1000IU) for infants (3).

1.9 Dietary vitamin D Intake in the UK

Dietary vitamin D intake across the UK is poor and often falls below the RNI. Nationally representative data for dietary vitamin D intakes in the UK have been described using data from the National Diet and Nutrition Survey (NDNS) rolling programme (136). The data summarised below are based on 4-day diet diaries collected from a sample of 3450 adults (19 years and over) and 3378 children (1.5 -18 years).

1.9.1 Younger adults

In the UK, mean vitamin D intake was below the RNI in 19-64 year olds at 2.8µg/day from food sources only and 3.9µg/day and 3.4µg/day from all sources in men and women, respectively (136).

1.9.2 Older adults

In the UK, mean vitamin D intake was below the RNI in those aged 65 years and over at 3.3µg/day from food sources only and 5.1µg/day and 5.2µg/day from all sources in men and women, respectively (136). For adults that were institutionalised, mean intake of vitamin D in men was 3.8µg/day from food sources and 3.9µg/day from all sources and in women vitamin D intake was 3.3µg/day and 3.4 µg/day from food sources and all sources, respectively (136).

1.10 Prevalence of vitamin D deficiency in the UK

Vitamin D deficiency is very common in the UK, with some sub-groups of the population who have an increased risk of deficiency and insufficiency.

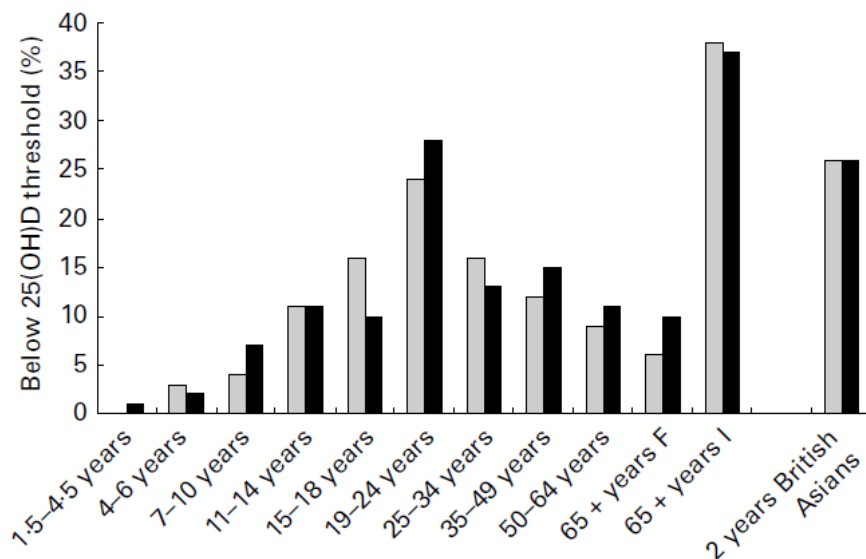
1.10.1 Younger adults

Men and women aged 19-64 years had a mean 25(OH)D concentration of 43.5nmol/l and 47.3nmol/l, respectively. Approximately 24% of men and 22% of women have a total 25(OH)D <25nmol/l (136).

1.10.2 Older adults

In adults aged 65 and over, the NDNS reports mean total 25(OH)D concentrations of 47nmol/l in men and 43nmol/l in women; with 16.9% of men and 24.1% of women with a plasma 25(OH)D concentration <25nmol/l throughout the year (136).

Figure 10: A summary of vitamin D deficiency (<25nmol/l) in the UK by age group



F, free-living; I, institutionalised; male, light grey bars; female, blackbars

Source: Lanham-New et al., (137)

1.10.3 Seasonal Variation in total 25(OH)D

Across all age groups, total 25(OH)D concentrations are lower in the winter (January-March) than summer months (July-September); 4-10 years: summer = 66nmol/l vs. winter = 37nmol/l; 11-18 years: summer = 52.3nmol/l vs. winter = 31.5nmol/l; 19-64 years: summer = 57.5nmol/l vs. winter = 34.8nmol/l; >65 years: summer = 50.5nmol/l vs. winter = 40.5nmol/l(3). In the UK, total 25(OH)D concentrations in Caucasians (n=125; age 20-60 years) are typically highest at the end of summer (September: ~71nmol/l) and lowest at the end of winter (February: ~46nmol/l). **Table 2** provides a summary of the proportion of the UK population with a total 25(OH)D of <25nmol/l in the winter and summer months (3).

Table 2: Prevalence of total 25(OH)D <25mmol/l by age group in the winter and summer months in the UK

Age group	Prevalence of deficiency by Season	
	Winter (%)	Sumer (%)
4-10 years	31	2
11-18 years	40	13
19-64 years	39	8
65+ years	29	4

Source: SACN (3)

1.11 Population sub-groups at risk of vitamin D deficiency/insufficiency

Several groups are at high risk of vitamin D deficiency and insufficiency in the UK. These include, pregnant and breastfeeding women; teenagers and younger adults, infants and young children under 5 years of age; older people aged 65 years and over; obese people; people who have low levels of exposure to the sun and people with darker skin (138).

In the UK, there is a high prevalence of vitamin D deficiency in younger adults and the elderly or institutionalised (137). Healthy younger adults appear to have a shorter 25(OH)D half-life and less dietary intake of vitamin D than healthy older counterparts, and this may contribute to lower levels of total 25(OH)D found in younger adults (Bowles et al., unpublished). The causes of vitamin D deficiency in older age groups are likely to be multifactorial. There is reduced dermal synthesis of vitamin D in response to sunlight (34). Older age groups are more likely to use high factor sunscreen (139), wear clothing that conceals the skin (30) and have lower levels of physical activity (140) which may lead to less time spent outdoors in comparison to younger age groups.

There is also a high prevalence of deficiency and insufficiency among ethnic groups in the UK, particularly those from South Asian and African-Caribbean origin (141). This is likely due to a combination of factors including, less sunlight exposure and wearing of religious or traditional clothing that block UVB sunlight (141) and darker skin pigmentation (33).

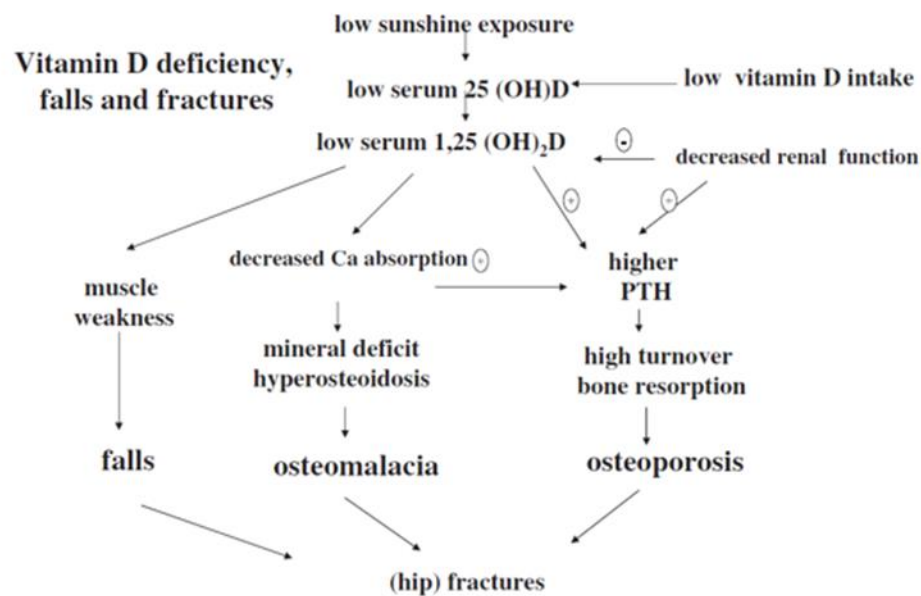
Vitamin D levels are typically lower in obesity, and this is likely due to a volumetric dilution effect (142, 143).

1.12 Consequences of vitamin D deficiency and insufficiency

Low total 25(OH)D levels in older adults are associated with adverse consequences for calcium metabolism, such as secondary hyperparathyroidism (144). Secondary hyperparathyroidism is the main cause of bone loss in vitamin D deficiency (145). However, very low levels of 25(OH)D (<25nmol/l) can impair calcium absorption (82) and cause low serum ionized calcium (81). This, in turn, is deleterious to bone health (**figure 11**).

During prolonged vitamin D deficiency, PTH remains elevated, stimulating bone turnover (145). High turnover bone contains high volumes of non-mineralised bone (osteoid bone). The lower mean osteoid age also contributes to high turnover bone containing less mineral. In severe vitamin D deficiency (<12.5nmol/l), > 5% of the bone tissue becomes osteoid bone, leading to osteomalacia (145). Lower total 25(OH)D has been associated with lower BMD and bone quality (146-148) and an increase in falls risk (149) and fractures (150) is also reported in older adults with serum 25(OH)D levels below 25nmol/l.

Figure 11: Vitamin D deficiency and the pathogenesis of falls and fracture



Source: Lips & van Schoor (145)

1.13 Free (unbound) vitamin D

In recent years, literature has speculated that 'free' or 'bioavailable' vitamin D concentrations may be a better biological marker of vitamin D sufficiency in certain conditions (151-153). The free hormone hypothesis suggests that only the unbound 'free' portion of protein bound hormones is biologically active, and that this should be measured for the accurate assessment of hormone availability (154). This concept is well-established for other hormones, such as thyroid hormones (155). Therefore, in the case of vitamin D, this suggests that only free 25(OH)D (or 1, 25(OH)₂D) may enter cells to exert a biological effect (156).

1.13.1 Transport of 25(OH)D and 1, 25(OH)₂D in circulation

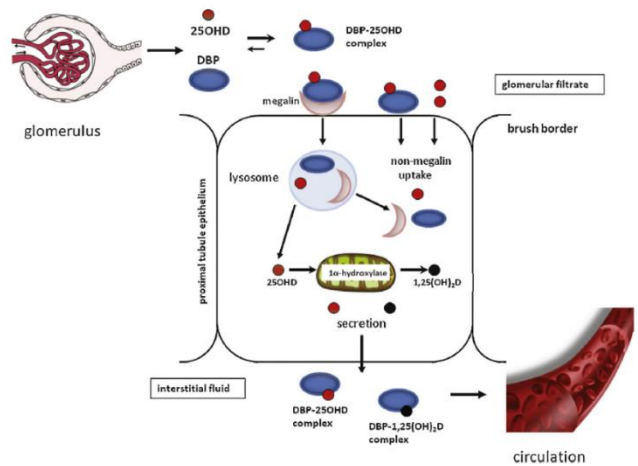
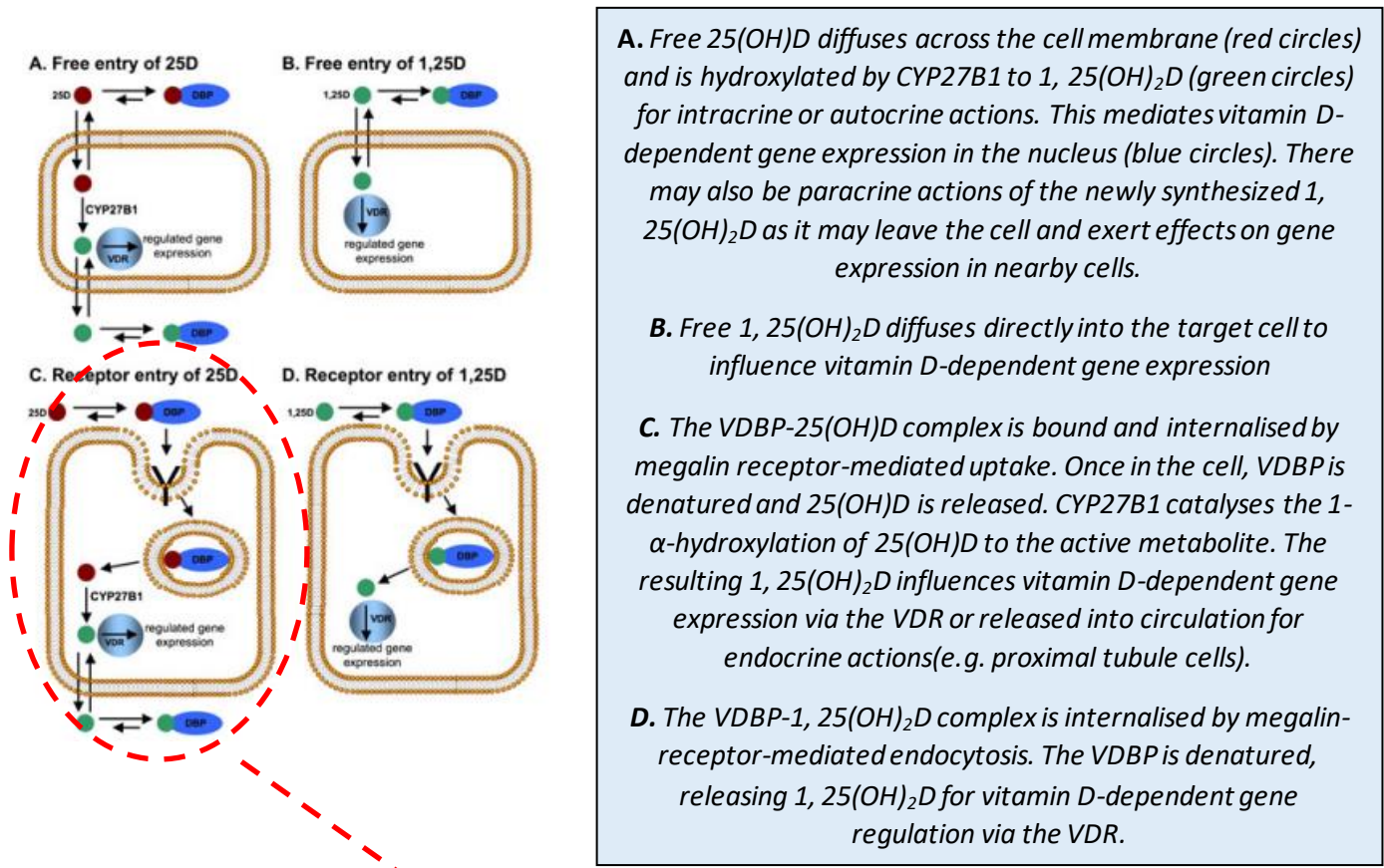
To understand this concept that free vitamin D metabolites may be more biologically relevant, we must first consider the transport of vitamin D metabolites (particularly 25(OH)D and 1, 25(OH)₂D) in circulation. In healthy individuals, approximately 85% of vitamin D metabolites are bound to VDBP in circulation, as VDBP has a relatively strong binding affinity for vitamin D metabolites (25(OH)D > 1, 25(OH)₂D > vitamin D₃) (157). Approximately 15% of vitamin D metabolites are more loosely bound to albumin (there is approximately a 1000-fold difference in binding affinity vs. VDBP) and a very small fraction of 25(OH)D (~0.03%) (157) and 1, 25(OH)₂D (~0.4%) (158) are free or 'unbound' in circulation. 'Bioavailable' vitamin D comprises of the free fraction and the fraction of vitamin D more loosely bound to albumin (i.e. approximately 15% in healthy individuals) (156, 157, 159), although there is debate over whether the bioavailable fraction is truly bioavailable to cells and this fraction is less well studied in association with health and disease outcomes (156, 158). Regardless, VDBP is a critical reservoir for circulating vitamin D metabolites, particularly for when vitamin D supply is low (156).

1.13.2 Cellular uptake of 25(OH)D and 1, 25(OH)₂D

Free 25(OH)D and 1, 25(OH)₂D are highly lipophilic molecules and can therefore passively diffuse across the cell membrane of target tissue cells (**figure 12**) (160). This mechanism occurs in most cell types (156). However, some tissue cells express the megalin/cubulin complex (a large transmembrane cell surface receptor complex) and have the ability to take up VDBP-bound 25(OH)D and 1, 25(OH)₂D. The best example of this mechanism is at the renal tubular cells which express the megalin/cubulin receptor complex, allowing for the internalisation of the VDBP and the bound vitamin D ligands (161). The high expression of CYP27B1 indicates that renal 1- α -hydroxylation is the main circulating source of 1, 25(OH)₂D and therefore this internalisation of the VDBP-25(OH)D is a pivotal component in the renal metabolism of 25(OH)D. Consistent with this, megalin knockout mice cannot internalise VDBP and its vitamin D ligand from the glomerular filtrate and as a consequence they have inadequate 1- α -hydroxylation of 25(OH)D and display rickets-like bone phenotype (162). Parathyroid cells also express megalin/cubulin and so a similar mechanism may operate in the parathyroid gland (158). Further work is needed to determine whether the expression or non-expression of megalin/cubulin in cells can distinguish between those

cell and tissue types that are responsive to VDBP bound metabolites and those cells that are only able to respond to free metabolites (163).

Figure 12: A summary of the different potential mechanisms for cellular entry of free 25(OH)D and 1, 25(OH)₂D and bound 25(OH)D and 1, 25(OH)₂D

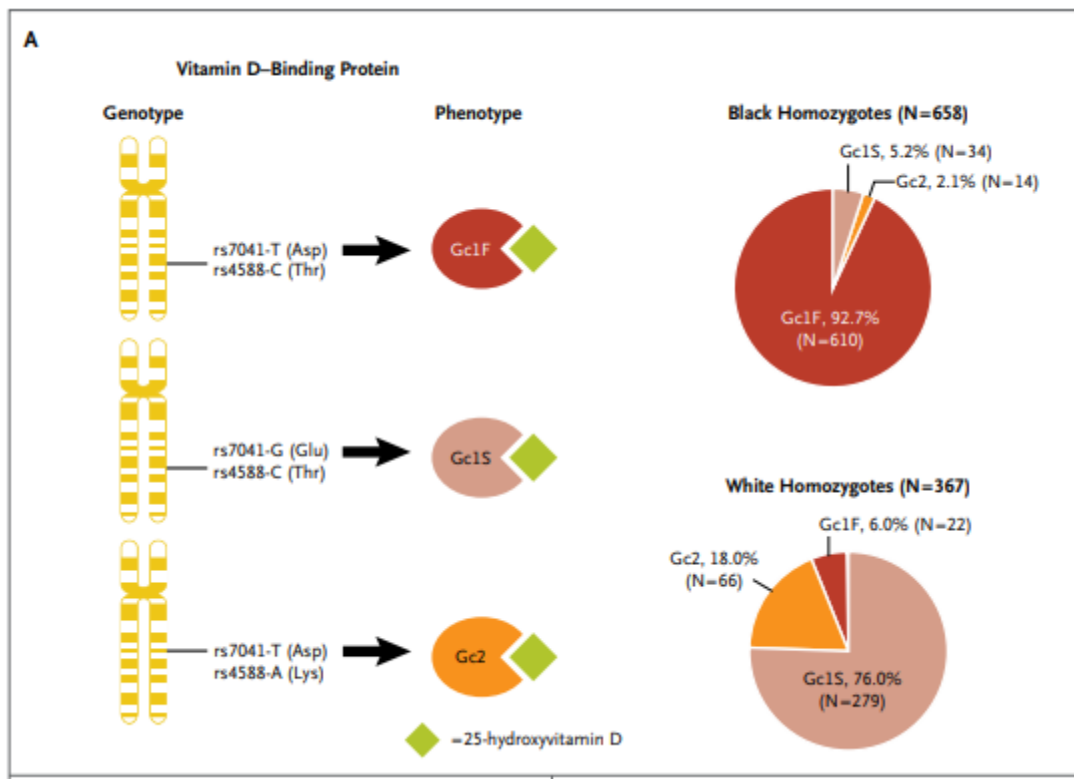


Source: Diagrams adapted from Chun et al., (160) & Chun et al., (164)

1.13.3 VDBP polymorphisms

The GC gene that encodes for VDBP is the most polymorphic gene known (156), with in excess of 1200 polymorphisms currently described (164). The three most common phenotypes are GC1f, GC1s and GC2 which are the result of unique combination of polymorphisms on the GC gene (rs7041 and rs4588). These polymorphisms result in differences in the amino acid sequence at positions 416 and 420, giving rise to these variant VDBPs (**figure 13**). Relative to the GC1F phenotype, the GC1S has a glutamic acid (E) instead of an aspartic acid (D) residue at amino acid position 416 and GC2 has a lysine (K) instead of a threonine (T) at amino acid position 420 (164). Therefore, if the 1F allele encodes the sequences for the protein, the amino acid sequence is **DATPT**, if the 1S allele encodes the sequence it is **EATPT** and if the GC2 allele encodes the sequence it is **DATPK** (156). There are also differences in the glycosylation of the GC1 and GC2 variants. The threonine residue at position 420 in the GC1 variant is glycosylated, but the lysine residue in the corresponding position in the GC2 variant is not (164). There are marked racial differences in the distribution of the GC genotype with large proportions of black populations having at least one GC1F allele (GC1F/GC1S, GC1F/GC1F & GC1F/GC2), whereas the GC1S allele is most common in white populations (**figures 13 & 14**) (151, 165).

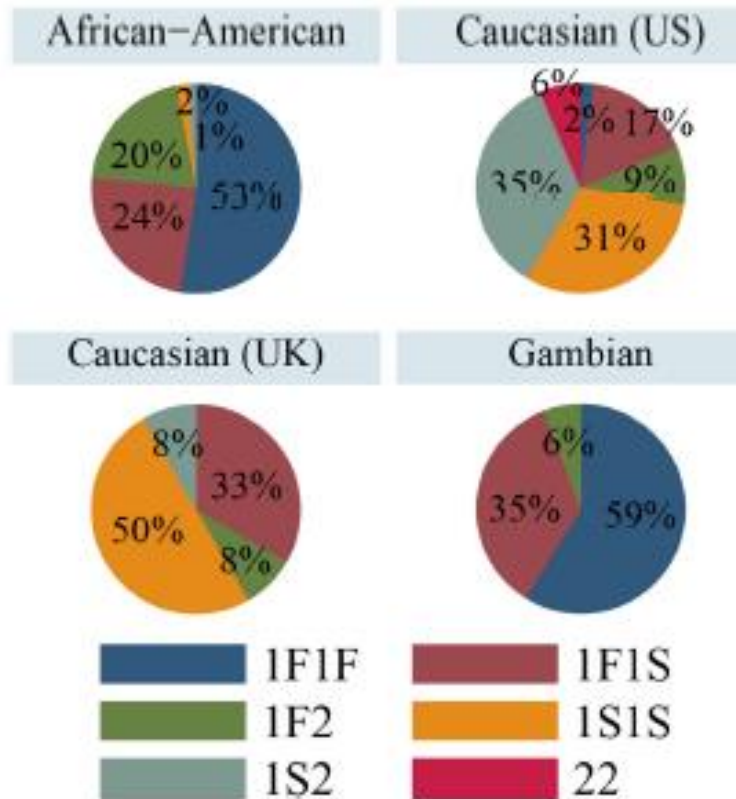
Figure 13: A summary of the different VDBP phenotypes and the frequency of these in community dwelling ($n = 2085$) black and white Americans.



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Source: Powe et al., (151)

Figure 14: A summary of the different VDBP genotypes by ethnicity from a US (MrOS) cohort (n = 101) African-American; n = 919 Caucasian), a UK cohort (n=12) and a Gambian Cohort (n=17)



Source: Nielson et al., (165)

1.13.4 Considerations for estimating free 25(OH)D and free 1, 25(OH)₂D

The method for calculating free 25(OH)D and free 1, 25(OH)₂D (see methods section for the calculated equations) rely on accurate measurements of VDBP and albumin and assumes that the binding affinity of VDBP and albumin for vitamin D metabolites is not invariant (163). However, several issues with VDBP and the assumptions of invariant affinity are apparent in the existing literature. There is wide variation in VDBP measurements using a range of measurement methods (165), apparent differences in affinity of the different VDBP alleles for vitamin D metabolites (166) and changes in binding affinity under certain pathological states and physiological conditions (156, 167). The method also relies on accurate measurement of total 25(OH)D, but this also varies widely depending on the measurement method used.

VDBP concentration in different VDBP alleles and implications for calculated free/bioavailable 25(OH)D research

It was initially reported that VDBP concentration differs by genotype. An observational study by Powe et al (151) reported that African Americans had lower VDBP levels (~50% lower) than Caucasians and so despite lower total 25(OH)D status in African Americans, the calculated bioavailable 25(OH)D did not differ by ethnicity. This suggested that in some population sub-groups free or bioavailable 25(OH)D might be a better marker of vitamin D status than total 25(OH)D in some ethnic populations.

However, more recently it has been demonstrated that there are methodological issues with the monoclonal antibody VDBP ELISA used by Powe et al (151) and that VDBP levels do not differ by race or genotype (165). In a large multi-ethnic and geographically diverse cohort of men, differences in VDBP by ethnicity were only reported when the monoclonal antibody was used in the assay (R & D Systems, USA) and not when measured using a polyclonal antibody ELISA or a directly measured ELISA method (165). These findings have been replicated in other studies and confirm that there are no significant ethnic disparities in VDBP concentration by assays using polyclonal antibodies (168) and LC-MS (168-170). It is hypothesised that there are selective affinity differences of the monoclonal ELISA for specific VDBP genotypes, with a lower affinity for GC1F and a higher affinity for GC1S (171). Therefore, calculated free 25(OH)D concentration is incorrectly elevated in those carrying the GC1F allele (due to a lower measured VDBP) and incorrectly lower (due to a higher measured VDBP) in those with the GC1S allele. Racial differences in VDBP concentration can therefore be explained by racial differences in VDBP genotype and associated differences in VDBP concentration when a monoclonal VDBP ELISA is used (163, 165, 171).

VDBP binding affinity for vitamin D metabolites

Calculated methods for free and bioavailable vitamin D metabolites also assume that each VDBP allele has the same binding affinity for vitamin D metabolites. However, differences in affinity for vitamin D metabolites have been found, but the rank order of the affinity of the different alleles for vitamin D metabolites is controversial (156). Earlier studies have reported that the GC1F allele has the greatest affinity for vitamin D metabolites and the GC2 the weakest affinity (166). However, Jones et al have reported that participants who were homozygous for the GC1F allele have the shortest 25(OH)D half-life in circulation, which would indicate a lower affinity for 25(OH)D compared to the other alleles (130). More recently, it has been reported that the lowest percentage free 25(OH)D is in the GC1S/1S haplotype and the highest in the GC1F/1F haplotype, with GC2/GC2 haplotypes in the mid-range despite similar VDBP concentrations (167). This would indicate that the GC1S allele has a greater affinity for 25(OH)D than the GC1F allele, with the GC2 allele somewhere in the middle (167). There is a need for research to further elucidate the exact differences in binding affinity between the different VDBP alleles.

Changes in VDBP binding affinity in different pathological conditions

Conditions (such as liver disease or pregnancy) that alter VDBP concentration or VDBP or albumin affinity for vitamin D metabolites may alter free 25(OH)D concentrations, the percentage of free 25(OH)D or the relationship between free and total 25(OH)D concentrations (156). Free 25(OH)D may therefore be a better measure of vitamin D status than total 25(OH)D in these conditions.

1.13.6 Free 25(OH)D vs. total 25(OH)D

Most studies that have compared free and total 25(OH)D and their association with calcium metabolism and non-calcitropic outcomes have used the calculated method to assess free 25(OH)D (163). A large proportion of these studies are limited by their use of the monoclonal antibody assay to measure VDBP and other factors affecting the calculated method as previously described. Some studies report stronger associations for free 25(OH)D with PTH, BMD and other non-skeletal health outcomes, whereas others do not (163). Findings are inconsistent, regardless of the method used to assess free 25(OH)D and whether the cohorts under study are racially homogenous (163). Further research is required on the assessment of free 25(OH)D vs. total 25(OH)D across a wide range of health outcomes using methods that are not confounded by VDBP genotype, such as the use of polyclonal VDBP assays and direct measurement of free 25(OH)D. Potential differences between different organ systems should also be investigated, with particular focus on those tissues with and without the megalin internalisation mechanism. Little attention has also been given to free 1, 25(OH)₂D. Free 1, 25(OH)₂D may be more important than free 25(OH)D as it is strictly regulated through endocrine feedback mechanisms (163).

1.14 Treatment of vitamin D deficiency

1.14.1 Testing for vitamin D deficiency

According to the Royal Osteoporosis Society (ROS) Clinical guidelines, serum 25(OH)D measurements and treatment should be considered in patients with bone diseases which may be improved with vitamin D treatment or where correcting a vitamin D deficiency is essential prior to beginning a specific treatment. For example, in patients with osteomalacia, correction of vitamin D deficiency would improve symptoms (e.g. calcification of bone, muscle weakness, musculoskeletal pain) or in osteoporosis before a patient starts treatment with an anti-resorptive drug (e.g. bisphosphonates or denosumab) to prevent hypocalcemia (131).

Symptoms of vitamin D deficiency/insufficiency are non-specific (e.g. muscle weakness, fatigue, chronic pain) and it may be difficult to ascertain whether they are due to low 25(OH)D and in such individuals a 25(OH)D measurement may be made (131). In individuals who are asymptomatic with no evidence of bone disease, then a 25(OH)D measurement is not recommended (131).

1.14.2 Thresholds for treatment

Current treatment of low levels of vitamin D depends on the severity of deficiency/insufficiency. Treatment is recommended in patients with a serum 25(OH)D < 30nmol/l (131). Treatment is also advised in patients with a serum 25(OH)D between 30-50nmol/l if they are at increased risk of fragility fracture, are taking antiresorptive medication for bone disease, have symptoms of vitamin D deficiency, are at increased risk of developing vitamin D in the future, have elevated PTH or suffer from conditions associated with malabsorption (131). For those patients with a serum 25(OH)D > 50nmol/l, lifestyle advice on maintaining adequate vitamin D levels should still be given (131).

1.14.3 Treatment regimes

The ROS clinical guidelines suggest that the key aims for correction of deficiency are to; use an adequate dose to raise 25(OH)D above 50nmol/l, to reverse clinical consequences in a timely manner and to avoid toxicity (131). Despite no adherence issues with intramuscular (IM) administration, oral administration is recommended due to the slower onset of repletion with IM (172, 173). Most standard treatment regimens, where rapid correction of deficiency is required (e.g. in symptomatic patients or patients about to start antiresorptive treatment), is based on fixed loading doses followed by a maintenance dose (131).

Loading doses are typically around 300 000IU and can be given as a weekly or daily split dose, depending on local availability of vitamin D preparations (e.g. 50 000IU once per week for 6 weeks, 20 000 IU given twice-weekly for 7 weeks or 800IU five times per day for 10 weeks) (131). Maintenance doses of 800-2000IU (depending upon whether the patient is at high risk of future deficiency) are typically considered at 1-month post loading dose (131).

1.15 Efficacy of standard dose (daily) vitamin D supplementation

1.15.1 Total 25(OH)D response to supplementation

There is a heterogeneous response of total 25(OH)D to vitamin D supplementation due to a wide range of physiological factors. The presence of fat in the lumen of the intestine is required for efficient absorption of vitamin (42), there is a blunted response to supplementation with higher BMI (174) and hepatic hydroxylation becomes saturated at increased concentrations of vitamin D in circulation (approximately 15nmol/l and the equivalent of 50µg/day), indicating that the response of total 25(OH)D to vitamin D supplementation is not linear at higher vitamin D intakes (175). Despite this, estimates in healthy adults range between a 0.53nmol/l and 0.7nmol/l increase in total 25(OH)D for every additional 1µg of vitamin D ingested (176, 177).

1.15.2 PTH response

Meta-analyses have established that daily vitamin D supplementation significantly decreases PTH in most vitamin D supplementation trials, particularly in people who are vitamin D deficient at baseline (124, 178-180). However, a total 25(OH)D status of <30nmol/l is not always associated with a compensatory rise in PTH and total 25(OH)D and PTH does not always respond to supplementation (181-184).

Factors affecting PTH response to supplementation

There are a wide variety of reasons that might explain why PTH does not always respond to vitamin D supplementation, including duration of treatment, calcium intake, baseline 25(OH)D status, baseline PTH, achieved change in total 25(OH)D, age and BMI (178, 180).

Treatment duration

The best effect of treatment on PTH response may be observed in trials that are greater than 12 months in duration (180), however no difference in treatment duration was found between PTH responders and non-responders to vitamin D supplementation in an earlier systematic review (178).

Dietary calcium intake

The response of PTH to vitamin D supplementation may also be modulated by dietary calcium intake and combined supplementation with vitamin D. A higher treatment effect has been observed in people with combined treatment compared to vitamin D monotherapy (180), suggesting that suppression of PTH may not be ensured without adequate calcium intake. In support, Bjorkman et al, (178) report that calcium was less frequently combined with vitamin D supplementation in PTH non-responders compared to patients in intervention groups who had a decrease in PTH levels (47.3% vs. 88.6%, $p < 0.001$).

This may be more relevant when vitamin D status is very low as there may be less active transport of calcium and so an individual may have to meet their dietary calcium needs by ensuring a high throughput of calcium. Indeed, the interaction between baseline 25(OH)D and dietary calcium intake has been explored. In studies where participants were vitamin D insufficient, calcium intake did not affect PTH response to vitamin D supplementation. However, in studies where participants were 25(OH)D sufficient at baseline, dietary calcium intake was inversely related to PTH (180).

Vitamin D dose and baseline 25(OH)D

It has been reported that participants with an insufficient baseline 25(OH)D (<50nmol/l) show a greater reduction in PTH than in participants with a sufficient baseline 25(OH)D (>50nmol/l) (180). However, Bjorkman et al, (178) report in their meta-analysis that approximately 300 (4.8%) PTH non-responders from intervention groups in 72 trials, did not have a significantly different baseline 25(OH)D status to responders, but did have a significantly lower baseline PTH and an overall smaller 25(OH)D response to supplementation.

It may be in some instances that the dose of supplementation is not high enough to elicit a PTH response. In the meta-analysis by Moslehi et al, (180) the mean level of supplementation in those trials in which PTH responded to supplementation was 57µg/day and mean baseline 25(OH)D was ~62.5nmol/l, whereas in the trials where PTH did not respond the mean level of supplementation was 30.5 µg/day and the mean baseline 25(OH)D was ~41.5nmol/l. This is in line with reports from Cranny et al who suggest that the reason for a lack PTH response may be due to a relatively low dosage for populations with a low baseline 25(OH)D. It may also be that changes in PTH may not occur with a baseline 25(OH)D concentration higher than the thresholds for PTH suppression.

Age

It has also been reported that the treatment effect of vitamin D on PTH is lower in people aged greater than 50 years compared to those who are aged lower than 50 years (180). However, in contrast, Bjokman et al (178) suggest that PTH non-responders were significantly younger than responders (56.6 years vs. 72.3 years, $P < 0.001$), although they did report an inverse association between age and PTH ($r = -.476$, $p < 0.001$). There are a number of plausible mechanisms as to why the 25(OH)-PTH axis may be disturbed with increasing age, meaning that for any given concentration of 25(OH)D PTH is higher with increasing age (178, 185), including poorer renal function (185) and intestinal 1, 25(OH)₂D resistance in older adults (186, 187).

1.15.3 The effect of vitamin D supplementation on BTMs

Studies investigating the effect of vitamin D supplementation on BTMs have had mixed findings and the effects are unclear. Vitamin D supplementation has had no effect on BTMs in some intervention studies. Vitamin D supplementation of 2800IU/day for 8 weeks had no significant effect on BALP, CTX, OC or PINP in hypertensive individuals (188). There was also no change in BTMs after 0, 5, 10 or 15µg of wintertime vitamin D in younger (20-40 years) and older (aged >64 years) (183). In addition, supplementation of younger adults (aged 18-27) with 15µg/day (and 1500mg of calcium) for 8 weeks in the winter period did not affect BTMs compared to placebo (181).

However, other studies have shown decrease in bone turnover markers. There was a significant decrease in PINP, but no change in CTX reported after 4-months of 20 000IU/week of vitamin D compared to placebo in middle-aged healthy adults (189). Furthermore, in a 6-month intervention study throughout winter in healthy men (aged 21-49 years), vitamin D supplementation decreased bone formation, but not bone resorption (190). Vitamin D supplementation was shown to significantly increase BALP concentration, but not other BTMs in younger vitamin D deficient Chinese adults (mean age 31 years) (191). Nine-months of vitamin D supplementation of 25µg/day in post-menopausal women (aged 50-65 years) demonstrated a significant reduction in PINP (13.4%, $P < .003$) and CTX (24.2%, $P < .001$), with no variations in BTMs observed in the placebo group (192).

1.15.4 The effect of vitamin D supplementation on BMD and fracture risk

Intervention studies to determine the effect of vitamin D and/or calcium supplementation on BMD and fracture incidence have had mixed success (145).

A systematic review investigating the effect of vitamin D supplementation on BMD, that included 23 trials with a range of baseline 25(OH)D concentrations, found no effect on BMD at the spine or total hip (193). However, they did report a significant 0.8% increase in femoral neck BMD (193).

In another meta-analysis of 23 trials (n=41419), vitamin D supplementation was associated with a reduced bone loss at the spine (1.19%; 0.76, 1.61; $P < .001$) and at the hip (0.54%; 0.35, 0.73; $P < .001$) (194). In the same meta-analysis vitamin D treatment was associated with a 12% reduction in fractures of all types. The fracture risk reduction was 24% greater in trials with better compliance. The effect of vitamin D supplementation was also greater when co-administered with calcium doses of ≥ 1200 mg than with doses that were < 1200 mg (0.80 vs 0.94; $P = .06$) and when vitamin D doses were > 800 IU compared to < 800 IU (0.84 vs 0.87; $P = .03$) (194).

A meta-analysis investigating the pooled effect on vitamin D supplementation on non-vertebral fractures (12 RCTs, n=42 279) and hip fractures (8 RCTs, n=40 886) in > 65 year olds found a 14% reduction in the risk of non-vertebral fractures (RR: 0.86, [95% CI: 0.77, 0.96]) (195). There was no significant reduction in the risk of hip fracture (RR: 0.91, [95% CI: 0.78, 1.05]). However, the pooled relative risk was improved when restricted to studies that had administered > 10 µg/day, which showed a 20% (RR: 0.80, [95% CI: 0.72, 0.89]) reduction in the risk of non-vertebral fracture and an 18% reduced risk of hip fracture (RR: 0.82, [95% CI: 0.69, 0.97]) (195).

Furthermore, in a trial sequential analysis (designed to reduce the risk of false positive type 1 errors) of 12 trials (n=27 834) vitamin D alone did not reduce hip fracture by 15% or more (196). However, there was heterogeneity in findings between trials that administered vitamin D monotherapy compared to trials that administered vitamin D and calcium. Vitamin D and calcium dual therapy did reduce the risk of hip fracture in institutionalised adults (2 trials, n =3853), but did not reduce the risk of hip fracture by $> 15\%$ in community dwelling adults (196).

1.16 Rationale for bolus dose vitamin D supplementation to treat vitamin D deficiency

Daily dosing regimens are problematic for some sub-groups at risk of deficiency, particularly older people.

Observational studies have also reported poor self-adherence to vitamin D and calcium supplementation (6, 7). In a cross sectional, observational study of adherence to treatment in 630 post-menopausal women (aged 64.1 \pm 8.7 years) in primary care for osteoporosis and who took calcium and vitamin D supplements, 36.2% (95% CI: 32.4, 39.9) had problems with tolerability, only 20.5% (95% CI: 17.3, 23.6) had a positive attitude towards treatment and only 50.0% (95% CI: 46.1, 53.9) had good self-reported adherence to treatment (6).

Randomized controlled trials have often reported poor compliance with daily dosing regimens (194, 197, 198). The average adherence rate to daily calcium and vitamin D supplements in a six-month placebo-controlled clinical trial of 107 older patients of mixed ethnicity (Mean age: 76.2 SD: +/- 5.6) was just 77.8% (SD = 26.1) and only 60.7% were at least 80% adherent (198). A meta-analysis of calcium and vitamin D supplementation trials reported that only 8 out of 15 RCTs had compliance rate of >80% and that those trials with higher compliance showed a significantly greater fracture risk reduction than did those with lower compliance rates (194).

1.16.1 Factors attributed to poor adherence to daily vitamin D supplementation

Factors attributed to non-compliance in older people include uncertainty about instructions and poor communication between prescriber and patient, cognitive and visionary impairment, dexterity issues, lack of social support, poor relationships with healthcare providers, poor educational attainment and forgetfulness (199). Other studies of vitamin D supplementation have demonstrated that ethnic minority participants and those with lower socioeconomic status have lower adherence rates than Caucasians and those of higher socioeconomic status (198). Higher household income and more years of education are also significantly associated with calcium and vitamin D adherence rates (198). Poor adherence has also been attributed to difficulty in swallowing combined calcium and vitamin D tablets, gastrointestinal side effects (198) and attitude towards vitamin D supplements and the number of concurrent treatments that a patient is taking (6).

Larger monthly, quarterly or annual doses have sometimes been advocated for the treatment of deficiency where standard dose supplementation is not practical. These larger dose preparations significantly reduce the burden of daily supplementation and address compliance issues in a cost-effective manner and reduces the likelihood of the target group remaining below the total 25(OH)D threshold for insufficiency (25(OH)D <50nmol/l). Large oral bolus doses also significantly increase total 25(OH)D in a more rapid manner than daily dosing strategies (200, 201).

1.17 Evidence of harm with higher total 25(OH)D concentrations

It has been demonstrated that vitamin D deficiency is associated with a wide range of disease outcomes and the therapeutic effects of vitamin D supplementation for a range of health and disease outcomes have been investigated. Despite this, there are also studies that have demonstrated that high 25(OH)D concentrations might be associated with adverse outcomes (202-209) and that in some instances large dose vitamin D supplementation may cause adverse events (8-10).

1.17.1 Observational studies demonstrating evidence of harm with higher 25(OH)D concentrations

Although the majority of observational studies demonstrate beneficial or neutral effects of vitamin D supplementation, there may be a U- or J-shaped association between total 25(OH)D concentration and the risk of harm. Reported harms associated with high total 25(OH)D levels include; frailty (204), schizophrenia (205), cancer (202, 203, 207-209) and overall mortality (206).

Inconsistencies between the findings of studies examining 25(OH)D in health and disease outcomes may be partly explained by differences in the populations under study, inappropriate sample sizes, the heterogeneity of measurement methods used for total 25(OH)D, the total 25(OH)D thresholds used to define vitamin D status, definitions of measure of the health or disease outcome, or residual confounders and differences in adjustment (or lack of adjustment) for potential confounders.

There are also several common limitations with observational studies. Observational studies are usually exploratory in nature and cause and effect cannot be established by such study designs. Observational studies are usually adjusted for multiple confounding variables, but they cannot eliminate the possibility of residual confounding factors. Although there are limitations with observational studies, they provide evidence that caution is needed before increasing 25(OH)D level too high and beyond the recommended safe limits and they also drive hypotheses for further investigation.

A summary of observational studies that have demonstrated harm with higher 25(OH)D levels can be seen in **table 3**.

Table 3: A summary table of observational studies that have demonstrated harm with higher concentrations of total 25(OH)D

Author	Study design	Sample	Study outcome	Total 25(OH)D method	Study Limitations & comments
Ensrud et al., (2010) (204)	Observational: Cross-sectional and longitudinal study of the association between baseline 25(OH)D level and incidence of frailty	n = 6307 Caucasian women aged ≥69 years (USA Study of Osteoporotic Fractures) Mean age 76.7 (SD: 4.8) years	Compared with the referent group (25(OH)D= 50-74.8nmol/l), the odds of frailty were higher in those with 25(OH)D > 75nmol/l (OR: 1.32 [95% CI: 1.06, 1.63]).	LC-MS/MS	This was an older Caucasian population Longitudinal findings were null Only measured 25(OH)D at baseline
McGrath et al., (2010) (205)	Population based case-control study	n = 424 participants with schizophrenia and n= 424 matches without schizophrenia recruited from Danish national health registers and neonatal biobank.	Neonates in the highest quintile of 25(OH)D ₃ (25(OH)D ₃ >51nmol/l) had a significantly increased risk of schizophrenia	LC-MS	
Michaelsson et al., (2010) (206)	Community based longitudinal study of adult men used to investigate the association between 25(OH)D and mortality	n = 1194 men ~12.7 years follow-up (Swedish Uppsala Longitudinal Study of adult men) Mean age at baseline ~71 years	U-shaped association between vitamin D concentrations and total mortality ~50% higher mortality rate in lowest 10% (25(OH)D <46nmol/l) and highest 5% (25(OH)D >98nmol/l) of 25(OH)D concentrations Cancer mortality higher at lower 25(OH)D concentrations (HR: 2.20; 95% [CI: 1.44, 3.38]) and high concentrations (HR: 2.64 [95% CI: 1.46, 4.78])	HPLC-MS	During follow-up 49% of participants had died A single measurement of 25(OH)D may not reflect long-term vitamin D status

Table 3: Summary table of observational studies that have demonstrated harm with higher concentrations of total 25(OH)D

Author	Study design	Sample	Study outcome	Total 25(OH)D method	Study Limitations & comments
Chen et al., (2007) (203)	Prospective case-cohort study of serum 25(OH)D concentration and risk of oesophageal and gastric cancers	Pretrial 25(OH)D measured in n = 979 cases diagnosed with cancer during 5.25 years follow-up vs. n = 1105 controls study was nested within 29 584 healthy adults aged 40–69 years Linxian (China)	Increased risk of developing oesophageal cancer in men for those in the highest quartile of 25(OH)D concentration at baseline (HR: 1.77 [95% CI: 1.16, 2.70, <i>P</i> = .0033)	IDS Immunoassay	Prospective analysis no association found in women Only measured 25(OH)D at baseline
Ahn et al., (2008) (202)	Prospective case-control	n = 749 cases vs. n = 781 controls. 25(OH)D assessed from blood drawn at least 8 years prior to diagnosis of cancer. Study nested within the Prostate, Lung, Colorectal and Ovarian Screening Trial	25(OH)D concentrations greater than the lowest quintile were associated with an increased risk of aggressive disease indicating that higher circulating 25(OH)D concentrations may be associated with increased risk of aggressive disease	RIA	The highest quintile (71.8-129.5nmol/l) did not show an increased OR of aggressive disease vs. Q1: ORs: Q2 vs Q1: 1.20 [95% CI: 0.80, 1.81]; Q3 vs Q1: 1.96 [95% CI: 1.34, 2.87]; Q4 vs Q1: 1.61, 95% [CI: 1.09, 2.38] and; Q5 vs Q1: 1.37 [95% CI: 0.92, 2.05; <i>P</i> trend = .05)
Tuohimaa et al., (2004) (209)	Longitudinal nested case-control study	n = 622 cases & n = 1451 matched controls data from men who donated blood for BioBanks in Sweden, Norway & Finland	Low (25(OH)D <19nmol/l) and high (25(OH)D ≥80nmol/l) associated with a higher risk of developing prostate cancer	RIA	

Table 3: A summary table of observational studies that have demonstrated harm with higher concentrations of total 25(OH)D

Author	Study design	Sample	Study outcome	Total 25(OH)D method	Study Limitations & comments
Stolzenberg-Solomon et al., (2006) (208)	Case-control study of 25(OH)D concentration and association with pancreatic cancer risk	n = 200 cases and n = 400 matched controls Study used pre-diagnostic 25(OH)D and was nested within the Beta Carotene Cancer Prevention cohort of male Finnish smokers, ages 50 to 69 years at baseline	Higher vitamin D concentrations (25(OH)D >65.5nmol/l) associated with a 3-fold increased risk of pancreatic cancer vs. lower concentrations (25(OH)D <32nmol/l) (OR: 2.9 [95% CI: 1.6, 5.5]; P trend = 0.001)	RIA Diasorin	A follow up study by the same group did not confirm these findings in men and women aged 55-74 years. The highest (25(OH)D >82.3nmol/l) vs. lowest quintile (25(OH)D <45.9nmol/L): OR: 1.45 [95% CI, 0.66, 3.15]; P trend = 0.49)(210)
Stolzenberg-Soloman et al., 2010) (207)	Pooled nested-case control study of participants from 8 cohorts within the Cohort Consortium Vitamin D Pooling Project of Rarer Cancers (VDPP) (1974–2006). The aim was to evaluate whether pre-diagnostic 25(OH)D was associated with pancreatic cancer risk	n = 952 cases of pancreatic adenocarcinoma with a median follow up of 6.5 years vs. 1333 matched controls	A high 25(OH)D concentration (≥ 100 nmol/L) was associated with a significant 2-fold increase in the risk of pancreatic cancer overall vs. 50-75nmol/l (OR: 2.12 [95%CI: 1.23, 3.64).	RIA Diasorin	Controls were matched to each case by cohort, age, sex, race/ethnicity, date of blood draw, and follow-up time. Positive association might be a statistical artefact due to the selective choice of thresholds for 25(OH)D (211).

1.18 Efficacy of oral bolus dosing on biochemical parameters of calcium and vitamin D metabolism

A limited number of studies have been published that have investigated the effect of a single oral bolus dose of vitamin D₃ on biochemical parameters of calcium and vitamin D metabolism. A summary of published studies is found in **chapter 4**. It is clear that a single large bolus dose of cholecalciferol is effective at rapidly increasing serum total 25(OH)D concentrations across many population sub-groups (172, 212-219).

1.19 RCTs demonstrating potential adverse events after Bolus dosing - falls and fracture

In some instances, the safety of high vitamin D bolus doses has been brought in to question. In one randomised-placebo controlled trial, 2317 community dwelling older women (mean age 76 years) were treated with a single oral bolus dose of 500 000 IU or placebo, annually for 3 years (8). Averaged over 3 years, the treatment group had significantly more falls; 74% compared with 68.4% on placebo ($P < 0.003$) (falls rate RR: 1.15, [95% CI 1.02, 1.30], $P = 0.03$) and a 26% higher rate of fractures (RR: 1.26, [95% CI; 1.00, 1.59], $P = .047$) (8). The higher incidence rate of falls was particularly marked in the three-month period after each dose (RR: 1.31, [95% CI; 1.12, 1.54], $P = .001$) (8). The study population was older women considered to be at high risk of hip fracture at baseline. In addition, the baseline 25(OH)D was 53nmol/l in the treatment group, which is considered sufficient without requirement for treatment.

In another large double-blind randomised controlled trial of community dwelling elderly men and women (median age: 79, IQR: 77-83) in the UK, an annual intramuscular 300 000IU bolus of vitamin D₂ was compared to placebo (9). There was no difference in the number of falls between groups, but hip fractures were 80% higher in women in the treatment group (HR: 1.80, [95% CI: 1.12, 2.90]; $P < .02$) (9). Colles fractures were 34% higher (HR: 1.34, [95% CI: 0.91, 1.98]), but this was not statistically significant (9). In men, the number of fractures did not differ between groups.

In an RCT of 3717 care home residents (mean age: 85 years) treated with a 3-monthly bolus of 100 000IU of vitamin D₂ compared to no vitamin D, there was no significant difference in the incidence of fracture, but the non-vertebral fracture rate between the groups was non-significantly higher in the treated group (RR: 1.48, [95% CI: 0.99, 2.20]) (220).

Lower doses of vitamin D, administered more frequently have also recently been associated with higher rates of falls in an elderly population (10). Older men and women ($n = 200$, mean age 78 years) with a prior fall were randomised to receive either: 60 000IU of vitamin D₃ monthly, 24 000IU vitamin D₃ monthly + 300ug of 25(OH)D daily, or 24 000IU vitamin D₃ monthly (control/standard treatment equivalent to 800IU/day) (10). The falls rate in the highest treatment dose groups (60 000IU: 66.9% [95% CI: 54.4, 77.5]; 24 000IU + 25(OH)D: 66.1% [95% CI:

53.5, 76.8]) was significantly higher than the 24 000IU (47.9% [95% CI: 35.8, 60.3]) group (P=0.048) (10). In addition, participants reaching the highest quartile of total 25(OH)D (110-243nmol/l) at 12-month follow up had a 5.5-fold higher (95% CI: 2.1, 14.5) odds of falling compared to those reaching the lowest quartile (52.3-74.5nmol/l)(10). There were also fewer falls in the lower 25(OH)D replete range (mean 0.84 falls) than in the higher 25(OH)D replete range (mean, 1.59 falls) (10). These findings suggest that there may be a therapeutic 25(OH)D range for falls prevention.

In support of a therapeutic 25(OH)D for falls prevention, Gallagher (221) reports data from a 1-year double blind placebo controlled trial in community dwelling women (mean age 67 years). His group found that fall rates were higher in the placebo and lower dose D₃ treatment groups (400 & 800IU daily) and in the higher dose groups (4000IU & 4800IU daily) than in the middle dose groups (1600IU, 2400IU & 3200IU daily)(221). For the 4000IU and 4800IU treatment groups combined the falls rate was 68.6% vs 27.3% in the 1600IU and 3200IU treatment groups combined (221). The total 25(OH)D level associated with the increased falls rate was in the upper quartiles (90-165nmol/l) (221), similar to that found by Bischoff-Ferrari et al (10). However, the lowest faller rate was in the 2000IU treatment group, whereas in the study by Bischoff-Ferrari et al the faller rate was highest in the 60 000IU treatment group, which is equivalent to 2000IU per day (10). This would indicate that it is perhaps the bolus dosing regimen which may be harmful when compared to daily dosing regimens.

1.20 Potential Mechanisms of increased falls risk

1.20.1 Increased participation in “at risk” falls behaviour

It has been hypothesized that an increase in falls and fracture after bolus dose vitamin D supplementation may be the result of participants feeling better and engaging in more “at risk” of falls behaviour due to the benefits of vitamin D supplementation (222). However, in the Australian study where there was an increase in falls and fracture in the 3-month period after administration of an annual 500 000IU dose of vitamin D₃ (8), there were no significant differences between groups in any measured outcomes of mental health and well-being (223). There were also no between-group differences in the circumstance or activity surrounding the fall events (224). Similarly, an increased opportunity to fall did not explain findings by Bischoff-Ferrari et al (10).

1.20.2 Vitamin D physiology in response to excess vitamin D in circulation

As vitamin D supply increases, the rate of 25-hydroxylation also increases in the liver to produce 25(OH)D (225). It is thought that the rate of 25-hydroxylation is proportional to the available amount of vitamin D₃ and that this reaction is not saturable (225), although at higher vitamin D inputs total 25(OH)D production does seem to plateau (175, 177). As serum 25(OH)D increases, more 1, 25(OH)₂D is produced per unit of 1- α -hydroxylase enzyme within

the kidney (226). As 25(OH)D levels increase, the serum/plasma concentration of 1, 25(OH)₂D is kept relatively constant due to the upregulation of the catabolic 24-hydroxylase degradative pathways (68, 227). Therefore, cholecalciferol treatment shifts vitamin D metabolism toward net degradation through increments in FGF-23 and reductions in PTH (68). This in turn, stimulates CYP24A1 (51) and inhibits CYP27B1 activity at the kidney (57, 58). Despite this, as 25(OH)D concentrations continue to rise, a limit may be reached where the substrate-driven output of 1, 25(OH)₂D can no longer be regulated (225).

1.20.3 Vitamin D toxicity causes people to fall

The mechanism(s) by which high-dose vitamin D toxicity may occur, and potentially leading to falls and fracture, is currently unknown. It is postulated that increased concentrations of a vitamin D metabolite reach the VDR in the nucleus of target cells and cause increased gene activation. However, it is not clear which vitamin D metabolite, and exactly how it becomes elevated. The key hypotheses are considered below:

Excess total 1, 25(OH)₂D

One hypothesis is that there is overproduction of 1, 25(OH)₂D due to excessive precursor levels and this leads to overexposure of the VDR in target cells to the active vitamin D metabolite. Excess vitamin D entering circulation raises total 25(OH)D levels, increasing circulating total 1, 25(OH)₂D concentrations, which in turn increases intracellular 1, 25(OH)₂D concentrations of target cells (47). Excessive intracellular 1, 25(OH)₂D, could lead to adverse effects through VDR binding, such as hypercalcaemia.

There are many classical tissues in which 1, 25(OH)₂D acts, including in the intestinal tract, kidneys and bone. In the enterocyte cells of the intestine, 1, 25(OH)₂D enhances the absorption of calcium and phosphorus (71). In bone, 1, 25(OH)₂D stimulates osteoclast differentiation from stem cell precursors and increase osteoblast production of RANKL. Overall, this leads to an increase in bone resorption and a net release of calcium from bone and into circulation to raise blood calcium levels (227).

Excess free 25(OH)D

It is also postulated that excess vitamin D intake may raise circulating total 25(OH)D concentrations to levels that exceed the binding capacity of VDBP. This releases free 25(OH)D which can enter target cells and have a direct effect on gene expression (47).

During vitamin D toxicity, the overloading of VDBP by the many different vitamin D metabolites compromises the ability of VDBP to restrict entry of other vitamin D metabolites into the cell. Of these, the usually inactive metabolite 25(OH)D has the strongest affinity for the VDR and so it is plausible that at higher free concentrations this metabolite could also bind with the VDR and induce transcription (47). Therefore, of the hypotheses put

forward to explain the trigger event for toxicity, one of the most plausible is a disproportionate increase in the free concentration of 25(OH)D. When the 25(OH)D metabolite is unbound it can freely enter the cell and could bind to the nucleus and stimulate gene transcription. Alternatively, the free 25(OH)D may act as a substrate for excessive intracellular 1- α -hydroxylation.

Excess free 1, 25(OH)₂D

The excess vitamin D entering circulation is likely to cause a rise in the levels of many vitamin D metabolites, especially vitamin D₃ and 25(OH)D. In this hypothesis, the concentrations of these metabolites exceed the binding capacity of VDBP and cause the release of free 1, 25(OH)₂D (due to its relatively low binding affinity for VDBP compared to other metabolites) which can then enter target cells and interact with the VDR. The ability of VDBP to restrict entry of this metabolite may become impaired. In normal vitamin D physiology, the relative low affinity of 1, 25(OH)₂D for VDBP and the relative high affinity for the VDR make this the only metabolite with the ability to exert reasonable biological effect within the cell (47). In this scenario of toxicity, the excess free 1, 25(OH)₂D may induce gene expression via the VDR. Jones (2008) suggests that the most plausible hypothesis for an adverse event due to toxicity are increases in the free concentrations of 25(OH)D and 1, 25(OH)₂D as when they are unbound they can freely enter a cell, bind with the VDR and stimulate gene expression.

In support of this hypothesis, we know that there are certain conditions where elevated free 1, 25(OH)₂D is implicated in vitamin D toxicity. Sarcoidosis is one disease that can result in vitamin D toxicity, with evidence to show that the mechanism of toxicity involves elevated circulating levels of free 1, 25(OH)₂D. Also, in 11 patients with marked hypercalcaemia due to accidental repeated ingestion of a veterinary vitamin D concentrate, despite extremely elevated serum total 25(OH)D levels (847-1652nmol/l), only three had elevated total 1, 25(OH)₂D levels. However, percentage free 1, 25(OH)₂D was elevated in all nine of the patients in which it was measured and actual free 1, 25(OH)₂D was elevated in six out of those nine (228). These findings would indicate that there may be a role for elevated free 1, 25(OH)₂D in the pathogenesis of hypercalcaemia in vitamin D toxicity.

Short-term protective upregulation of CYP24 (25-hydroxyvitamin D-24-hydroxylase)

Another hypothesis is that any adverse effects of a large bolus dose of vitamin D may be triggered by a compensatory upregulation in the enzyme responsible for the catabolism of 1, 25(OH)₂D (CYP24), resulting in decreased levels of 1, 25(OH)₂D in the blood and tissues (222). Lower 1,25(OH)₂D levels could, in theory, decrease the amount of calcium available to muscle cells, negatively influencing muscle cell contraction and relaxation and subsequent global muscle function (229). This is supported by an animal study that showed that a diet of excess vitamin D leads to an increase in renal and intestinal CYP24 activity, indicating an enhanced metabolic clearance of 1, 25(OH)₂D (230).

However, human RCTs to date do not demonstrate a fall in $1, 25(\text{OH})_2\text{D}$ level after bolus dose supplementation. For example, after IM administration of 600 000IU of ergocalciferol there was a 37.5% increase in circulating $1, 25(\text{OH})_2\text{D}$ at 4 months from baseline (9). In another bolus dose supplementation study in older adults, $1, 25(\text{OH})_2\text{D}$ levels increased at 3 days and only fell to baseline levels at 90 days after oral administration of a single 600 000IU dose of cholecalciferol (214). There is a paucity of data from human studies that have performed serial assessment of $1, 25(\text{OH})_2\text{D}$ after bolus dose vitamin D supplementation, and further studies should include measurement of this metabolite.

1.20.4 A model of vitamin D toxicity – the consequences of a disproportionate rise in free vitamin D metabolites

As discussed, it has been proposed that the binding capacity of DBP may be overwhelmed in some situations. In reference to the study by Sanders et al (8), Heaney proposed that the increased rate of falls in the treatment group was because of vitamin D toxicity in the initial period after administration due to the binding capacity of DBP being overwhelmed by the large increase in cholecalciferol (231). This led to a sharp rise in total $25(\text{OH})\text{D}$, with a relatively greater increase in free $25(\text{OH})\text{D}$ (231). In addition, because $1, 25(\text{OH})_2\text{D}$ has the weakest binding affinity to DBP of the vitamin D metabolites, free $1, 25(\text{OH})_2\text{D}$ may also rise. Despite this very few studies have measured free $25(\text{OH})\text{D}$ and free $1, 25(\text{OH})_2\text{D}$ after oral bolus dosing. There may be several possible consequences of a disproportionate increase in these free vitamin D metabolites.

Hypercalcemia

The higher free $25(\text{OH})\text{D}$ and/or free $1, 25(\text{OH})_2\text{D}$ results in greater calcium absorption and this would manifest as a rise in serum calcium and urinary calcium. Adults are usually in mineral balance and therefore hypercalciuria may be indicative of excess vitamin D (225). This can be easily monitored by measuring the calcium/creatinine ratio in a 24-hour urine sample. For those with normal renal function, hypercalciuria is defined as a urine calcium/creatinine ratio of $>1 \text{ mmol/mmol}$ ($>0.37\text{mg/mg}$) (225). The key criterion for vitamin D toxicity is hypercalcemia because this is what causes actual symptoms (225). Symptoms of hypercalcemia can include fatigue, weakness and muscle pain. Hypercalcemia may also induce changes in mental status including confusion and disorientation. Any of these symptoms could explain an increase in falls risk.

Direct effects of free metabolites on the brain

There may be direct effects of the free $25(\text{OH})\text{D}$ and/or free $1, 25(\text{OH})_2\text{D}$ on the brain. Animal studies have demonstrated that the vitamin D receptor (VDR) is expressed in the developing and adult brain of rats (232) and hamsters (233). The first study to show the distribution of the VDR, and the $1\text{-}\alpha\text{-hydroxylase}$ enzyme responsible for the formation of the active vitamin D metabolite, in the human brain was presented by Eyles et al (234). The presence of a functional receptor in the brain may not be of importance if the $1, 25(\text{OH})_2\text{D}$ ligand cannot access

the brain. Therefore, it is also important to consider the prospect of the ligand being synthesised locally in the brain by the 1- α -hydroxylase enzyme, catalysing the conversion of 25(OH)D to 1, 25(OH)₂D.

Eyles et al show that the VDR and 1- α -hydroxylase are present in diverse regions in the human brain and this indicates a diverse role for vitamin D in the brain (234). This work has been disputed due to the non-specificity of the commercial antibodies used for detection of the VDR in immunohistochemistry studies and other studies have found no evidence of the VDR in the brain (63) or muscle (110). Despite this, confirmation of VDR protein in the brain of rats has been established recently using proteomic techniques and mass spectrometry (234).

The VDR is intensely expressed and the 1- α -hydroxylase moderately expressed in the granule cell layer of the cerebellum (101). The cerebellum is essential for balanced muscular activity. Therefore, this region of the brain may be of interest when considering the effects of vitamin D toxicity on the brain and understanding the mechanisms behind why toxicity may cause falls. Any disproportionate rise in free 25(OH)D and/or free 1, 25(OH)₂D may have an adverse effect on this brain region that could interfere with motor function and lead to an increased risk of falling.

Direct effects of muscle

Excess free vitamin D metabolites could have a direct adverse effect on muscle function through “excess binding” with the VDR in muscle, and this might explain the increase in falls after a large bolus dose. However, it is controversial as to whether the VDR is actually present in skeletal muscle cells, with some studies unable to detect the VDR (110), but other groups have documented the expression of the receptor in human muscle tissue (112, 235).

Despite the reported increase in fallers after a single large bolus of D₃ (8), other bolus dosing studies to date do not appear to show any detrimental effects on muscle strength and function. Quadriceps muscle strength and SPPB scores increased after a 600 000IU oral D₃ bolus in older adults (aged 65 years and over) (212). A 300 000IU dose of vitamin D₃ given orally or IM in community dwelling adults (aged >65 years), increased quality of life scores, decreases measure of pain and improved functional mobility (assessed by TUG) (236). However, in a 9-month RCT in 686 vitamin D sufficient community dwelling women (aged 76.7+/- 4 years), an oral 150 000IU D₃ bolus every three-months had no effect on grip strength or TUG (237). In addition, with monthly high dose oral vitamin D₃ there does not appear to be any negative treatment effect on muscle function test scores (e.g. SPPB), despite a reported higher incidence of falls with higher dose treatment (10).

The effect on blood pressure - Postural hypotension

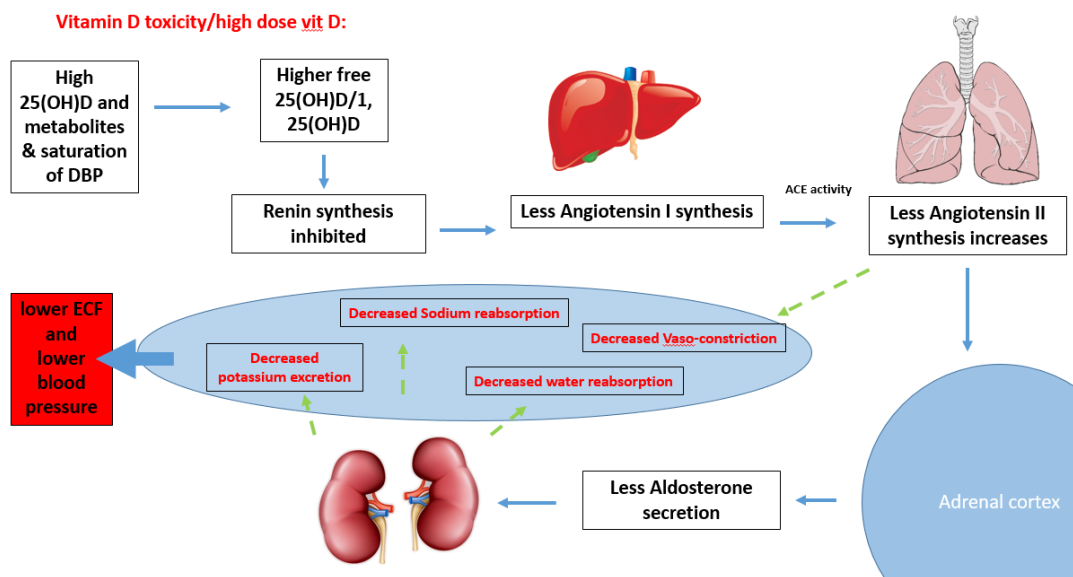
The Renin-Angiotensin System (RAS) is central to the regulation of blood pressure (238). There is ample evidence to demonstrate that vitamin D, particularly the active 1, 25(OH)₂D, is a potent suppressor of renin synthesis and therefore an important modulator of blood pressure.

Renin gene expression is suppressed by $1, 25(\text{OH})_2\text{D}$ (239) and mice lacking the VDR have elevated renin production and angiotensin II, causing high blood pressure (238, 239). In CYP27B1 knock out mice, levels of renin are elevated and mice have hypertension, which can be reversed with $1, 25(\text{OH})_2\text{D}$ treatment (240).

In clinical studies, inverse associations have been reported between $1, 25(\text{OH})_2\text{D}$ and blood pressure in normo- and hypertensive adults (241, 242) and total $25(\text{OH})\text{D}$ and renin activity and hypertension (243, 244). A recent meta-analysis also showed a significant reduction in systolic ($-0.102\text{mmHg} \pm 0.04$, [95% CI: $-0.20, -0.03$]) and diastolic blood pressure ($-0.07\text{mmHg} \pm 0.03$, [95% CI: $-0.14, -0.006$]) with vitamin D supplementation (124). Trials with doses $>100\mu\text{g/day}$ demonstrated greater reductions in systolic and diastolic blood pressure than those with doses $<100\mu\text{g/day}$ (124).

Therefore, we can postulate that after a single large bolus dose there may be postural hypotension. This may be due to the inhibition of renin synthesis at the kidney by excess free vitamin D metabolites, leading to reduced angiotensin I production. This, in turn, leads to less angiotensin II and less aldosterone secretion and ultimately a lower blood pressure (figure 15). Postural hypotension can cause dizziness or fainting, which could explain the increase in falls after a D_3 bolus.

Figure 15: An overview of the hypothetical mechanism for how a single bolus dose of D_3 could lead to postural hypotension



Source: Self-drawn by author

1.21 Summary of research gaps

No human bolus dosing study has previously set out to mechanistically understand vitamin D toxicity and therefore a holistic mechanistic picture is missing. In studies of vitamin D bolus dose supplementation to date there has been a lack of comprehensive and universal measurement of total 25(OH)D and other related biochemical parameters. There is heterogeneity in the bolus dose administered, measurement time points (peak levels of vitamin D metabolites are not always necessarily captured), study populations, biochemical measurements made and assays used, and baseline 25(OH)D status (a summary of these studies is given in **chapters 4 & 5**). This heterogeneity makes for difficult interpretation, particularly in understanding the potential mechanisms of adverse effects after a bolus dose.

Few studies have reported changes in total 1, 25(OH)₂D after a single, large bolus of vitamin D₃, the majority of which have been reported by the same research group. Free 25(OH)D in response to a large single bolus dose of vitamin D has only been reported on one occasion and this was in a small sample of healthy participants (n =29) and burns patients (n= 20) (245). This was estimated using calculated methods only, the drawbacks of which have already been described, and so no study has reported directly measured free 25(OH)D in response to a bolus dose of vitamin D₃. In addition, no study has previously reported free 1, 25(OH)₂D in response to a single large bolus dose of vitamin D₃. Furthermore, only one single bolus dosing study of vitamin D₃ has reported total 25(OH)D₃ as measured by LC-MS/MS; the gold standard measurement method. No previous study has reported the effects of a large bolus dose of vitamin D on postural changes in blood pressure, despite this being a plausible mechanism for causing falls. A comprehensive study is needed to further understand what changes occur (if any) to free vitamin D metabolites after a single, large vitamin D₃ bolus. It is important that these research gaps are addressed as healthcare practitioners need to be able to make informed decisions regarding the optimum safe bolus dose to give to achieve target 25(OH)D levels.

1.22 Study aims

1.22.1 Primary aims

This study will investigate changes in total and free 25(OH)D and total and free 1, 25(OH)D at 1-week, 4-weeks and 12-weeks post-administration of three different large single oral doses of vitamin D₃ (50,000IU, 150,000IU and 500,000IU) in vitamin D deficient (<30nmol/l) postmenopausal women. This will allow us to test the clinically important hypothesis that higher dose boluses of vitamin D₃ could cause a disproportionate rise in free 25(OH)D and/or 1, 25(OH)₂D, leading to hypercalcemia or other adverse health outcomes.

A greater understanding of changes to these metabolites after bolus dosing will further our understanding of the potential role they play in vitamin D toxicity.

1.22.2 Secondary aims

The study will also determine the effect of three different large single oral doses of vitamin D₃ on parameters of calcium metabolism, bone turnover, muscle function and blood pressure.

1.23 Research Questions

1. How do different bolus doses of vitamin D affect total 25(OH)D, free 25(OH)D and 1, 25(OH)₂D in vitamin D deficient postmenopausal women?
2. Is there a disproportionate rise in free 25(OH)D and or free 1, 25(OH)₂D compared to total 25(OH)D and 1, 25(OH)₂D after bolus dose vitamin D supplementation?
3. How do different bolus doses of vitamin D affect measures of calcium metabolism (including VDBP, Albumin, PTH, serum calcium, serum phosphate and 24-hour urinary calcium excretion) in vitamin D deficient postmenopausal women?
4. How do different bolus doses of vitamin D affect bone turnover markers (PINP, Osteocalcin & CTX) in vitamin D deficient postmenopausal women?
5. How do different bolus doses of vitamin D affect measures of physical function and muscle strength in vitamin D deficient postmenopausal women?
6. How do different bolus doses of vitamin D affect cardiovascular parameters in vitamin D deficient postmenopausal women?

1.24 Hypotheses

1. There will be a dose-response increase in total 25(OH)D, total 1, 25(OH)₂D, free 25(OH)D and free 1, 25(OH)₂D by 1-week after bolus dose administration
2. There will be a disproportionate rise in free 25(OH)D in response to the largest dose of vitamin D₃ (500 000IU) 1 week after administration
3. There will be a disproportionate rise in free 1, 25(OH)₂D in response to the larger bolus dose of vitamin D₃ (500 000IU) 1 week after administration
4. In the largest bolus dose (500 000IU) treatment group, the higher percentage free 25(OH)D/free 1, 25(OH)₂D may result in greater calcium absorption and this would be manifest as a rise in serum ionised calcium and urinary calcium excretion
5. There will be a dose-response decrease in PTH in response to bolus dose supplementation
6. There will be a dose-response rise in albumin and vitamin D binding protein after bolus dose supplementation
7. There will be a dose-response increase in PINP, osteocalcin and CTX in response to bolus dose supplementation

8. There will be an improvement in SPPB and grip strength scores in the lower dose groups, but the higher dose group will have a negative effect on SPPB and grip strength scores
9. There will be a dose-response decrease in systolic and diastolic blood pressure and ARR in response to bolus dose supplementation

Chapter 2:

Methods

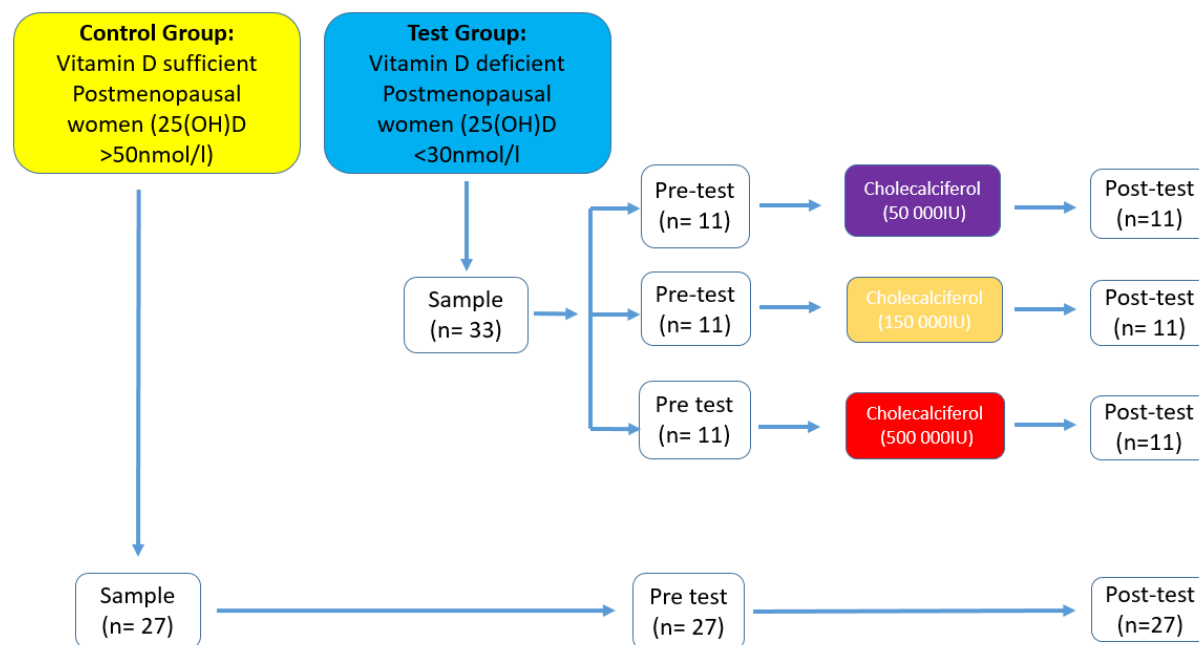
2.1 Study design

This was a single centre, double-blinded randomized controlled trial to determine the effects of three different bolus doses of vitamin D₃ on free 25(OH)D and total 25(OH)D in postmenopausal women.

Changes in total and free 25(OH)D and 1, 25(OH)₂D, bone biochemical and clinical response to three different large single oral doses of vitamin D (50,000IU, 150,000IU and 500,000IU) in 33 vitamin D deficient (<30nmol/l) postmenopausal women were studied over a three-month period (**figure 16**). In addition, a concurrent control group of 27 vitamin D sufficient (total 25(OH)D >50nmol/l) postmenopausal women were recruited.

These doses were chosen as we believed there may be a dose-response increase in free 25(OH)D relative to total 25(OH)D across the three doses. The doses are also representative of the spectrum of clinical practice in vitamin D supplementation. A single dose of up to 300 000IU is a recognized regime for vitamin D replacement and hasn't been associated with adverse side effects. One trial has indicated a possible increase in the risk of falls in the first 3 months after a 500 000IU bolus (8), and this observation led to the hypothesis that a high dose bolus could oversaturate VDBP. However, that study included women who were not vitamin D deficient. In the present study only women who are vitamin D deficient (25(OH)D <30nmol/l) received a vitamin D bolus dose. This has allowed testing of the dose-response of free 25(OH)D and free 1, 25(OH)₂D, with limited risk of toxicity.

Figure 16: An overview of the study design



2.2 Ethical considerations

Favorable opinion was received from the East Midlands-Nottingham 1 Research Ethics Committee (15/EM/0345) and local research governance approval was received from the STH research department.

All study participants gave written informed consent prior to enrolment on the study in accordance with GCP procedures. Security of research data was assured by acting in accordance with the 1998 Data Protection Act. All research personnel who were University of Sheffield personnel had Sheffield Teaching Hospitals Foundation Trust honorary contracts. The study was carried out in compliance with the research ethics committee approved protocol and in accordance with the Academic Unit of Bone Metabolism and Clinical Research Facility standard operating procedures.

Consent to use and store samples was obtained according to the Human Tissue Act 2004. On completion of the study, samples from adults were stored in the STH HTA licensed biorepository and adopted by the Musculoskeletal Biobank.

Overall risk to the participants during the study was minimal. Blood samples were taken by a trained phlebotomist. There are small risks from having blood samples taken, such as risk of bruising and infection. All participants receiving cholecalciferol were vitamin D deficient (<30nmol/l). Vitamin D was only administered to vitamin D deficient (<30nmol/l) participants to reduce the risk of toxicity. People with lower body mass may be at risk of vitamin D toxicity due to lower volumetric dilution (143). Therefore, a lower Body Mass Index (BMI) limit of 20kg/m² to further minimise the risk of any vitamin D toxicity. Participants were also otherwise healthy women who were not considered to be at high risk of falls (the previous study that reported an increase in falls after a large bolus dose of cholecalciferol included women at high risk of falls (8)).

2.3 Treatment groups

Serum free and total 25(OH)D was measured at baseline and 5(+/-2), 28(+/-3), and 84(+/-5) days after administration of 50 000IU, 150 000IU or 500 000IU of cholecalciferol to assess the effect of high dose supplementation on the ratio of free 25(OH)D to total 25(OH)D. PTH, 1,25(OH)₂D, serum calcium, ionized calcium, 24 hour urinary calcium, bone turnover markers and muscle function were also measured at baseline and 5(+/-2), 28(+/-3), and 84(+/-5) days after administration of the vitamin D.

2.4 Concurrent control group

The same measures were undertaken for control group participants but only at baseline and 84(+/-5) days. Controls did not attend for visits at 5(+/-2) and 28(+/-3) days. The control group was recruited to compare untreated levels of vitamin D across the study period to the treatment groups.

2.5 Primary research endpoint

- Within-group change in the ratio of serum free 25(OH)D to total 25(OH)D from baseline to 7 days (week 1)

2.6 Secondary research endpoints

- Within-group change in the ratio of serum free 1,25(OH)D to total 1,25(OH)D from baseline to 7 days
- Between-group difference in proportion of serum free 25(OH)D to total 25(OH)D at 7, 28, and 84 days
- Between group difference in endocrine biochemical measures at 7, 28 and 84 days
- Between group difference in different biochemical markers of bone turnover at 7, 28 and 84 days
- Between group difference in muscle function tests at 7, 28 and 84 days
- Between group difference in blood pressure markers of postural hypotension at 7, 28 and 84 days

2.7 Sample size

The primary outcome measure was change in free 25(OH)D at one week from baseline. No data were available to estimate the expected magnitude of change in this context, although this was very difficult to speculate. We designated 30% as a clinically significant change and used results from our previous studies of vitamin D physiology in obesity to determine that 30% would be approximately 1.3pg/ml. We assumed a standard deviation of 1.9pg/ml and that correlation between free 25(OH)D at baseline and visit three would be 0.7. To achieve 90% power for demonstrating this mean difference as statistically significant at the 2.5% two-sided level required 28 patients per group. Due to difficulties in finding eligible participants, and the necessary restriction to complete study visits during the winter, the final number recruited was below this target.

2.8 Inclusion Criteria and exclusion criteria

Participants were postmenopausal women who were vitamin D deficient (<30nmol/l) or vitamin D sufficient (25(OH)D >50nmol/l) at screening.

Participants were primarily recruited by invitation email sent to the University and NHS trust staff. Poster adverts were also placed around the University and hospital (Northern general Hospital, Sheffield, UK). Participants were also recruited by mail outs from GP surgeries where potentially eligible patients had been identified from the GP database. Participants were also recruited from the hospital biochemistry database, where appropriate patients who had recently been diagnosed as having a total 25(OH)D level were identified. Potential participants were also approached by invitation letter from the Academic Unit of Bone Metabolism volunteer database.

2.8.1 Inclusion criteria:

55 years and over

Study participants were postmenopausal women aged 55 years and over. Post menopause was defined as at least 5 years since their last menstrual period. This criterion helps to exclude perimenopausal and menopausal women who undergo alteration to their bone metabolism such as, increased bone resorption and decreased bone mass.

Caucasian

Only Caucasian individuals were recruited to avoid confounding due to ethnic differences in vitamin D metabolism. Vitamin D levels vary by ethnicity; in the UK, total 25(OH)D levels are higher in Caucasians than black and Asian ethnic groups (141) and white Americans have higher levels of total 25(OH)D than black Americans (151).

There are several different genetic types of VDBP, and that type varies between different ethnic groups (151, 160, 246). VDBP genotype may influence the binding affinity of VDBP to 25(OH)D and other vitamin D metabolites and therefore influence free 25(OH)D levels (247). Therefore, VDBP genotype is an important consideration when investigating free or bioavailable 25(OH)D and for the correct interpretation of results.

Body Mass Index between 20 kg/m² and 30kg/m²

People with lower body mass may be at risk of vitamin D toxicity due to lower volume of dilution (143). Therefore, a lower Body Mass Index (BMI) limit of 20kg/m² was set to minimise the risk of vitamin D toxicity. The dilutional model postulates that vitamin D₃ is in equilibrium between with VDBP and fat stores within the body at a ratio of approximately 1:12 (248). Vitamin D₃ after bolus dosing will not just be distributed into serum and bound to VDBP, but will also be distributed into the totality of the body fat and other body compartments (143). The higher the BMI, the less we would expect serum 25(OH)D levels to rise due to distribution in the greater mass of the different body compartments, particularly fat. An upper body mass index of 30kg/m² was therefore used to minimise the attenuating effect of BMI (or fat mass) on any rise in serum free and total 25(OH)D levels.

25(OH)D < 30nmol/l for treatment groups or 25(OH)D >50nmol/l for control group

One trial has indicated a possible increase in the risk of falls in the first 3 months after a 500 000IU of cholecalciferol (8). A separate study has shown that mean fall rate was higher at 12-months after monthly oral doses of 60 000IU of cholecalciferol than 24 000IU (10). Therefore, caution is required when administering bolus

doses of cholecalciferol to limit any potential adverse consequences. However, participants in these previous studies were not vitamin D deficient at baseline and administration of bolus dose cholecalciferol may have raised 25(OH)D levels too high in some participants. We administered the bolus doses of cholecalciferol to vitamin D deficient (<30nmol/l) participants only to limit the risk of 25(OH)D levels rising above a safe threshold.

Able and willing to participate in the study and provide written informed consent.

All participants were provided written informed consent prior to enrolment, in accordance with Good Clinical Practice guidelines and all participants were sufficiently mobile to carry out the SPPB.

2.8.2 Exclusion criteria:

History of any long-term immobilization

Defined as no weight bearing functionality for longer than three months. Immobilization causes changes to bone and endocrine factors that are critical to the outcomes of the study. Bone mineral density is lower at weight bearing sites after bed rest (249-251). There are also changes to bone turnover markers with immobilization; bone formation and resorption markers are elevated after 30 days of bed rest (249, 250), but appear to return to baseline levels after 1 year of recovery (250). Bed rest also appears to affect serum levels of total 25(OH)D, 1, 25(OH)₂D and PTH and urinary calcium excretion (249, 250).

High trauma fracture or low trauma fracture less than one year prior to recruitment

Bone remodeling is important for normal fracture repair and so a higher rate of bone turnover is associated with recent fracture. Bone turnover markers are elevated from 1-2 weeks post fracture (252, 253) and bone formation markers may remain slightly elevated up to one-year post-fracture (252, 254, 255). Fracture may also indirectly affect 25(OH)D level, other endocrine factors related to vitamin D metabolism and bone turnover, through altered levels of physical activity or immobilization. For example, total 25(OH)D level is reduced three-weeks post tibial and femoral shaft fractures in all age groups (256). PTH and 1, 25(OH)₂D were significantly elevated 3-months post-fracture compared to baseline in postmenopausal women who had suffered a hip fracture (257) and both markers have been shown to increase steadily between 3 and 365 days post fracture in Caucasian postmenopausal women (252).

Diabetes mellitus

People with Type II diabetes mellitus (T2DM) were excluded because the disease impairs bone metabolism, reduces skeletal quality and may increase fracture risk (258, 259). Several studies have shown that the impaired bone quality may be due to low bone turnover in T2DM compared to healthy controls and it has been consistently demonstrated that bone turnover markers are lower in T2DM patients (259-261). Individuals with a history of T2DM were therefore excluded due to the known impairments caused to bone health and effects on markers of bone turnover that are measured in this study.

History of diagnosed restrictive eating disorders

Low bone mineral density (BMD) has been described in restrictive eating disorders, such as Anorexia Nervosa (AN) and Bulimia Nervosa, and is caused by failure to accrue peak bone mass in adolescence and bone loss in young adulthood (262-264). Effects on markers of bone turnover and have been demonstrated, but with inconsistent results. Several studies have shown a decrease in bone formation and bone resorption markers when compared to healthy controls (262, 265). Other studies have demonstrated an uncoupling of bone homeostasis, characterised by an increase in bone resorption and a decrease in bone formation (263, 266, 267). Five days of low energy availability also resulted in increased bone resorption (PINP) decreased bone formation (β -CTX) and in women (268).

There are also uncertain effects on vitamin D metabolism in restrictive eating disorders. Total 25(OH)D (267, 269-271), free 25(OH)D (269) and PTH (267, 269, 271) in AN patients have been shown to be no different to healthy controls. However, a recent meta-analysis indicates that total 25(OH)D and 1, 25(OH)₂D are lower in unsupplemented AN patients compared to healthy controls, whereas vitamin D supplemented AN patients had significantly higher total 25(OH)D levels (272). It has been proposed that the regulatory mechanisms of the vitamin D endocrine system are altered in AN patients characterized by a lower 1,25(OH)₂D and higher 24,25-(OH)₂D concentration for a given total 25(OH)D and that this reflects a higher relative activity of 24-hydroxylase enzyme (270).

Individuals with a history of restrictive eating disorders were therefore excluded due to the known impairments caused to bone health the uncertain effects on vitamin D metabolism and bone turnover markers.

Use of medications or treatment known to affect vitamin D or bone metabolism

Individuals who used hormone replacement therapy for longer than ten years or within the year prior to recruitment were ineligible due to well established effects on bone turnover (273). Participants with a history of bisphosphonate use were also excluded due to known effects on bone turnover markers (274) and because those on bisphosphonates medication will already be taking vitamin D and calcium supplements. Those with a history of

glucocorticoid use were also excluded due to the known effects of the treatment on increasing bone resorption through extending the life of osteoclasts and reducing bone formation by inducing apoptosis in osteoblasts (275). Total 25(OH)D is also lower in those who use glucocorticoids and therefore there maybe wider implications for vitamin D metabolism (276). The use of asthma inhalers and steroids applied to the skin were not excluded as these are less likely to affect bone and vitamin D metabolism. Anticonvulsant use was ineligible due to effects on bone, calcium and vitamin D metabolism and falls risk (277).

Alcohol intake of greater than 21 units per week

Light to moderate alcohol consumption may be beneficial to overall bone health, resulting in higher bone mineral density and reduced age-related bone loss but excessive alcohol consumption has a deleterious effect on bone, and is associated with low bone mineral density, impaired bone quality and increased fracture risk (278, 279).

There are heterogeneous results from different cross-sectional studies to assess the association between alcohol consumption and vitamin D status (280). Alcohol consumption also appears to reduce levels of bone turnover markers in postmenopausal women (281, 282). Consumption of alcohol may also modulate the risk of falling. Whilst light consumption of alcohol may not influence the risk of falling or may even reduce the risk (283, 284), a high alcohol intake increases the risk of falling in older adults (283, 285).

Individuals with a high alcohol intake were therefore excluded due to the known impairments to bone health, the association with falling and the unknown effects on the vitamin D endocrine system and bone turnover markers.

Holiday with significant sunlight exposure in the last six weeks or planned sun holiday within study period or vitamin D supplementation >10µg/day

Vitamin D₃ is synthesized in the skin upon exposure to sunlight. Therefore, an individual is unlikely to be vitamin D deficient at screening after a recent sunlight holiday. Other vitamin D related endocrine factors may also be perturbed by recent sunlight exposure. Participants who were already taking a significant vitamin D supplement were excluded as they were also unlikely to be vitamin D deficient at screening.

History of or current conditions known to affect vitamin D or bone metabolism or abnormal clinical laboratory results

Participants with diagnosed conditions known to affect vitamin D or bone metabolism, including endocrine disorders (e.g. uncontrolled hyper-/hypo-thyroidism, hyperparathyroidism, hypo- or hyper-calcaemia), chronic renal disease, or malabsorption syndromes (e.g. Crohn's disease, Coeliac Disease or Inflammatory Bowel Disease) were excluded from the study. Participants with markedly abnormal clinical laboratory results at visit 1, as

assessed by the PI, were excluded. Participants were also excluded if they were currently participating in another clinical trial.

2.9 Study Procedures

Vitamin D metabolism is perturbed by sunlight exposure and so the study was conducted in winter months and early spring when sunlight in Sheffield is not strong enough for endogenous synthesis of vitamin D.

All participants were required to attend five visits to the Clinical Research Facility at Northern General Hospital (NGH), Sheffield, UK. At **visit 1**, participants gave informed consent and their eligibility was confirmed. Consenting participants were given 7-day diet diary instructions and 24-hour urine collection instructions and a sunlight exposure questionnaire to complete before their second visit. Baseline anthropometric measures, such as height and weight, were taken.

At **visit 2** participants were asked to attend for a fasted morning blood test. Participants returned their completed 7-day diet diary, sunlight exposure questionnaire and 24-hour urine collection. Treatment group participants were given a randomly allocated dose of liquid vitamin D (50 000IU, 150 000IU or 500 000IU). Participants completed a series of physical performance measures, as part of a Short Physical Performance Battery (SPPB) assessment, including a repeated chair stand test, a balance test and a short walk test. Participants also completed a grip strength test.

At **visits 3, and 4 and 5** participants returned their completed 24-hour urine collections. Fasted blood samples were taken. As per visit 2, participants completed the SPPB assessment. Participants completed a grip strength test.

Procedures conducted at each study visits are shown in **table 4** and a summary of participant involvement by study visit shown in **table 5**.

Table 4: Measurements/tests completed by participants at each study visit

Visit & Time point	Measurement
Visit 1	Written informed consent
Screening	7-day diet diary instructions 24-hour urine collection instructions. Sunlight exposure questionnaire. Blood sample for participants with unknown 25(OH)D status
Visit 2	Return of 24h urine collection for calcium and creatinine
Baseline (7-9 days after visit 1)	Return of 7-day diet diary. Anthropometric measurements (height & weight) Pulse and blood pressure Fasting blood sample for vitamin D and metabolites, PTH, serum calcium, bone turnover markers and other biochemical analysis Physical function tests: SPPB and grip strength. Administration of either: <ul style="list-style-type: none"> - 50 000IU of vitamin D₃ (Cholecalciferol) - 150 000IU of vitamin D₃ (Cholecalciferol) - 500 000IU of vitamin D₃ (Cholecalciferol) - No treatment for control group
Visit 3	Return of 24h urine collection for calcium and creatinine
(5±2 days after visit 2)	Pulse and blood pressure
Treatment groups only	Fasting blood sample for vitamin D and metabolites, PTH, serum calcium, bone turnover markers and other biochemical analysis Muscle function tests: SPPB and grip strength
Visit 4	Return of 24h urine collection for calcium and creatinine
(28±3 days after visit 2)	Pulse and blood pressure
Treatment groups only	Fasting blood sample for vitamin D and metabolites, PTH, serum calcium, bone turnover markers and other biochemical analysis Muscle function tests: SPPB and grip strength
Visit 5	Return of 24-hour urine collection
(84 ± 5 days after visit 2)	Return 7-day diet diary Pulse and blood pressure Fasting blood sample for vitamin D and metabolites, PTH, serum calcium, bone turnover markers and other biochemical analysis Physical function tests: SPPB and grip strength

Table 5: Summary of participant involvement by study visit

Study Visit	V1	V2	V3*	V4*	V5
Activity					
Informed consent	X				
Diet diary	X				X
Sunlight exposure questionnaire	X				
Height, weight & BMI	X				X
Blood for vitamin D screening if required	X				
Bolus vitamin D (If treatment group)		X			
Fasting blood sample		X	X	X	X
Short Physical Performance Battery		X	X	X	X
Grip strength test		X	X	X	X
24-hour urine calcium		X	X	X	X
Blood pressure		X	X	X	X

*Treatment group only

2.10 Intervention

The vitamin D₃ (cholecalciferol) was supplied in olive oil (dosing ampoules of 25 000IU in 1ml) by Consilient Health Ltd (UK). To maintain blinding of the patient and investigator, each participant received the same volume of olive oil:

- For the 50 000IU treatment, two x 25 000IU 1ml liquid ampoules were added to 18ml of olive oil to give 20ml of liquid in total
- For the 150 000IU treatment, six x 25 000IU 1ml liquid vitamin D ampoules were added to 14ml of olive oil to give 20ml of liquid in total
- For the 500 000IU treatment, 20 x 25 000IU 1ml liquid vitamin D ampoules were used to give 20ml liquid in total

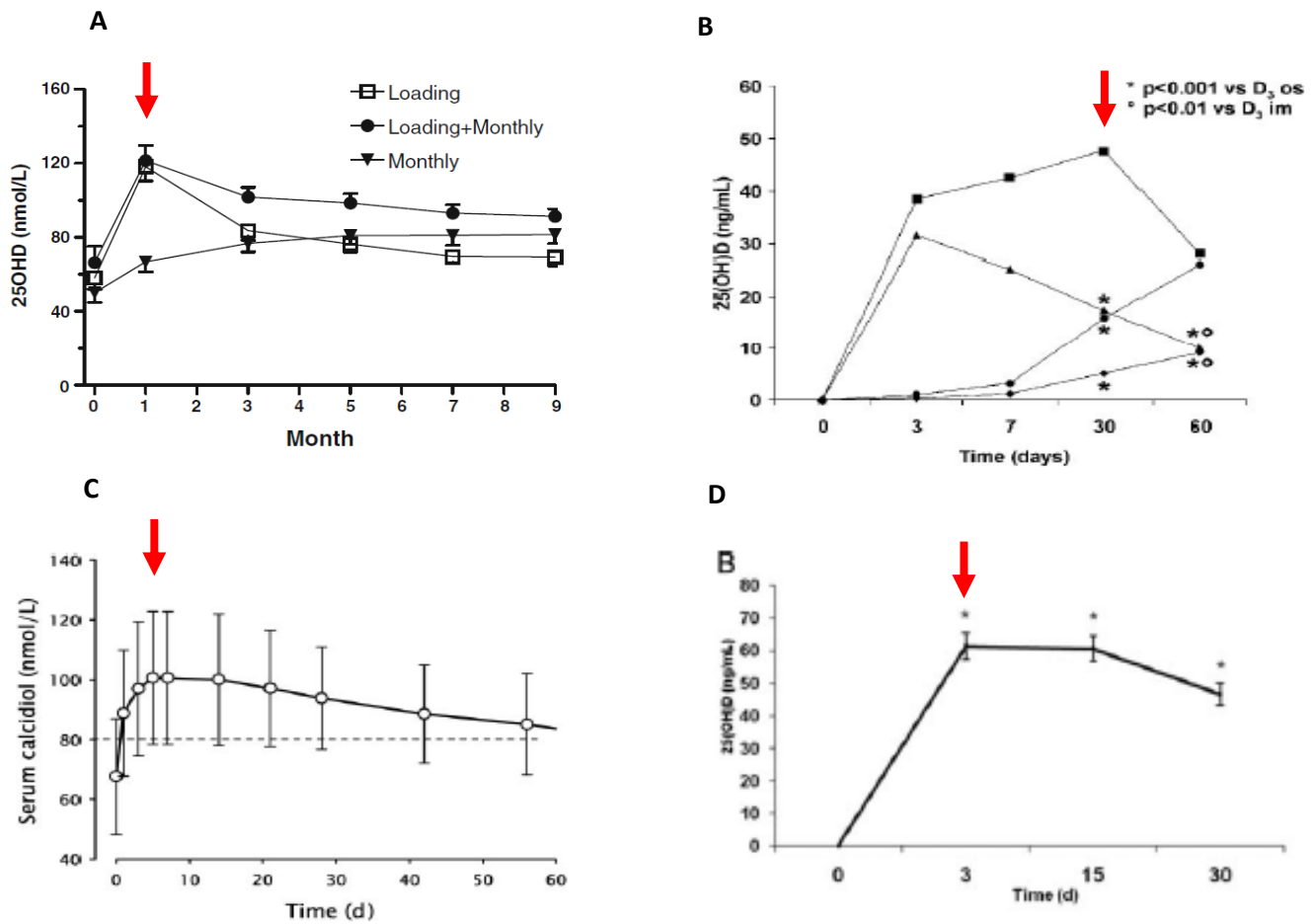
The vitamin D was stored by the pharmacy (NGH, UK). Ampules were supplied in batches on a weekly basis from pharmacy to a secure, temperature-controlled drug cabinet at the Clinical Research Facility (NGH, UK). Each participant's allocated dose was prescribed by a non-blinded study doctor, and prepared and administered by a non-blinded research nurse.

The treatment was poured onto three small pieces of bread so that the liquid was absorbed by the bread. Each participant was instructed to eat each piece of bread, ensuring that all liquid had been consumed. A small breakfast was then given to each participant to aid the absorption of the vitamin D. The breakfast consisted of toast with butter and a cup of coffee or tea with milk.

2.10.1 Justification of study time points

It is uncertain when peak vitamin D levels are reached after bolus dosing. The peak has been described at 3 days (214), 7 days (286) or 1 month (8, 172, 213) (**figure 17**). However, the study populations used in these studies are heterogenous and a range of bolus doses have been used. Therefore, to ensure that peak serum 25(OH)D was captured, follow-up time points were scheduled at 5+/- 2 days and 28+/- 3 days.

Figure 17: Serum 25(OH)D profiles after a vitamin D bolus dose from several studies. Red arrows indicate peak total 25(OH)D concentration



- A.** 63 healthy elderly participants (≥ 65 years) randomised to a 500 000IU loading dose vs. 500 000IU loading dose + 50 IU/month vs. 50 000IU/month (213).
- B.** Vitamin D repletion in 32 elderly female nursing home patients (aged 66-97 years). Single dose of 300 000IU cholecalciferol via oral or IM route vs. 300 000IU ergocalciferol via oral or IM route (172).
- C.** 30 healthy participants (aged 27-84 years) given 100 000IU cholecalciferol bolus (286).
- D.** Vitamin D repletion in vitamin D deficient 25–56 year-olds ($n=48$). Single oral dose of 600 000IU cholecalciferol (214).

2.10.2 Randomisation

In clinical trials with small sample sizes care must be taken to ensure that an unequal number of individuals are not assigned to each treatment arm as this would decrease the power to detect any statistically significant differences between groups (287). Block randomisation was used in this study to achieve an equal number of participants in each treatment arm. Two copies of the randomisation schedule were produced, one was kept in pharmacy and the other supplied to the unblinded nurse. A record of dosing allocation for each participant was kept by the non-blinded research nurse, and a copy of the prescription and administration record was returned to pharmacy. The participant, chief investigator and principal investigator were blinded to dosing to protect from researcher and participant bias.

2.11 Anthropometric measurements

Body height was measured in centimeters to the nearest 0.1 cm using a stadiometer and weight was measured in kg to the nearest 0.1 kg using an electronic balance scale. Participants were weighed and measured shoeless, but in lightweight clothing. Participants were asked to stand with their feet together and heels against the wall. Their head was positioned so that the Frankfort Plane was horizontal. BMI was calculated using Quetelet's index (weight (kg)/[height (m) squared]).

A single investigator carried out all anthropometric measurements to eliminate Inter-investigator variability.

2.12 Blood pressure & pulse

Vitamin D has potential effects on blood pressure (see chapter 1). Therefore, pulse and blood pressure were measured (lying and standing) with an automated sphygmomanometer (Dynamap).

2.13 Diet diary

Vitamin D metabolism is perturbed by dietary vitamin D intake and calcium intake and so these nutrients were assessed using an estimated seven-day diet diary. Although diet diaries can be burdensome to participants, they are the most accurate method to measure food intake in adults (288, 289) and are generally the gold standard method used in most nutrient validation studies (290). Diet diaries have been shown to be more strongly correlated with serum total 25(OH)D level than food frequency questionnaires (291).

The standardised diet diary was completed at baseline and before the final visit to check that dietary intake was consistent throughout the study. Data on supplement intake was also recorded. Participants were given a diet diary template and asked to record, in as much detail as possible, all food and drink items consumed over a seven-day period. Participants were also administered a food portion booklet. This booklet contained photographs of many common foods and illustrates different portion sizes of these foods. Participants were instructed to identify

which photograph best represented their portion size for a meal or snack consumed. Participants could also describe portion size in other measures, such as approximate weights or household units. Participants were asked to note on their diet diary any recipes used to make a meal, in as much detail as possible. Participants had a debriefing session with a nutritionist to improve the quality of dietary assessment (292). The data on nutrient intake collected was entered and analyzed using Windiets (Robert Gordon University, UK).

2.14 Sunlight exposure questionnaire

Habitual sunlight exposure was estimated using a sunlight exposure questionnaire at baseline. Due to a large number of influencing factors quantifying sunlight exposure is extremely difficult (13) and so there is no standardised questionnaire. A questionnaire was supplied by Professor Sue Lanham-New; Head of Department of Nutrition and Metabolism at the University of Surrey that has previously been used.

The questionnaire was scored as follows:

- For each month of the year participants were asked to score how often they are usually outside and exposed to the sun. A score of 3 for 'often', 2 for 'occasionally', and 1 for 'seldom'.
- The score given was multiplied by the total body areas exposed in each month by using the following from the rules of nine to estimate the surface area of the skin exposed to sunlight:
 - Head = 9% (front and back together)
 - Both Arms = 9% (L) + 9% (R)
 - Both Legs = 18% (L) + 18% (R)
 - Torso = 18% (front) + 18% (back)
 - Groin = 1%

For example, a participant ticking 'occasionally', and 'head' and 'arms' would get a score for that month of $(2 * (0.09 + 0.09 + 0.09)) = 0.54$. These scores were added together to give a 'total sunlight exposure' score.

2.15 Short Physical Performance Battery (SPPB)

The SPPB consists of three objective tests that assess lower body function. The method was derived from large epidemiological studies on healthy older adults (293). The SPPB has been used extensively in community dwelling older adults to assess functional health and is a significant predictor of falls (294), subsequent disability (293, 295) and mortality (293, 296) in this population.

The three SPPB tests are as follows:

2.15.1 Repeated chair stand test

The repeated chair stand test measures ability to stand from a chair without using arms, reflecting the strength of the legs. Participants were asked to sit on a chair with their feet placed on the floor, squarely in front of them. Their knees flexed slightly (at > than 90°) so that their heels are closer to the chair than the back of their knees. Participants kept their arms folded across their chest for the duration of the test. Participants were asked to stand and sit once to familiarise themselves with the procedure and to determine their capability to continue to the main part of the test. Participants were asked to, as quickly as possible, stand and sit five times continuously. The time taken for the participant to complete the 5 stand-and-sit cycles was recorded to the nearest one hundredth of a second. If the participant was unable to complete the test in full then this was recorded, giving the number of completed cycles, if any. The test was repeated twice and an average time derived.

There grading for the repeated chair stand test was as follows:

0 = unable to complete

1 = >16.7 sec

2 = 16.6-13.7 sec

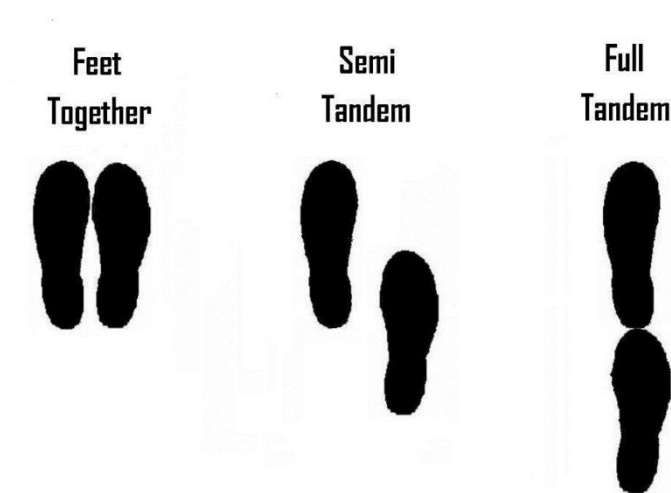
3 = 13.6-11.2 sec

4 = <11.1 sec

2.15.2 Balance testing

To assess balance, participants were instructed to stand unaided with their feet in three different positions, holding each position for a maximum of 10 seconds (**figure 18**). Participants were able to hold on to the researcher's arms to initially gain balance. Participants could move their arms, body and bend their knees to maintain balance but they could not move their feet. If the participant was able to hold the position for 10 seconds, then they progressed to the next stand position.

Figure 18: Feet positions for balance tests



An overall score for balance was derived:

0 = Feet together held for 0-9 seconds or unable

1 = Feet together held for 10 seconds, but <10 seconds on semi-tandem

2 = semi-tandem held for 10 seconds, but 0-2 seconds full-tandem

3 = semi-tandem held for 10 seconds, but 3-9 seconds full-tandem

4 = Full-tandem held for 10 seconds.

2.15.3 Eight feet walk course (2.44 metres)

A 2.44 metres long walk course was marked out (**figure 19**). Participants were asked to walk at their usual pace from the start point to the other end of the course (2.44 metres). Participants completed the test three times and an average time calculated.

A gait score was calculated as follows:

0 = unable to complete

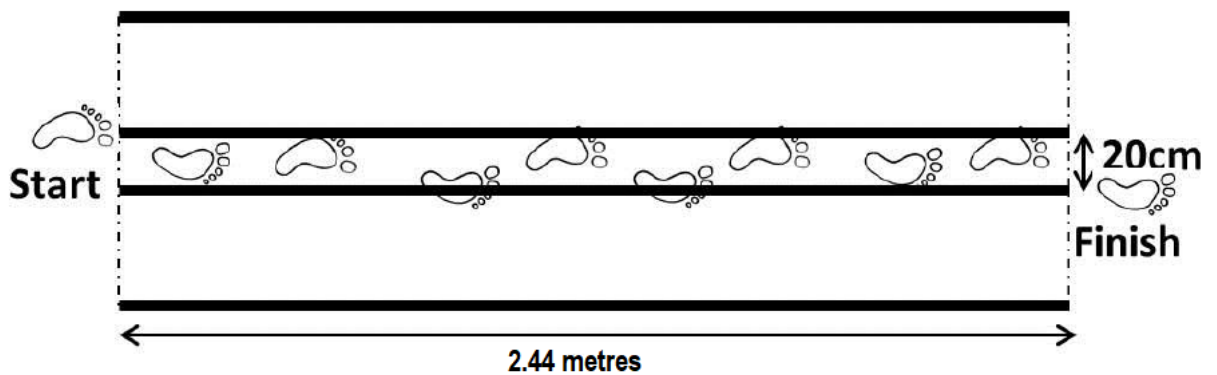
1 = >5.7 seconds

2 = 4.1-6.5 seconds

3 = 3.2-4.0 seconds

4 = <3.1 seconds

Figure 19: The 2.44 metres narrow walk course



Walking aids were not used during any of the SPPB tests. The participants were instructed to wear comfortable shoes and shoes with minimal or no heels were not worn to minimise the effect of footwear during the test.

All SPPB tests were carried out at baseline (visit 2) and visits 3, 4 and 5. When completed, a score between 0 (worst performance) and 12 (best performance) was calculated by totaling the scores from each of the three SPPB tests.

2.16 Grip strength

A digital hand dynamometer (Seahan Corp, Masan, Korea) was used to measure hand grip strength. This determined the strength of the hand and forearm muscles and acted as an indicator of neuromuscular function across the study time points. Participants were seated with feet flat on the floor. They were instructed to hold the dynamometer with their upper arm in line with their body. The forearm was held at approximately 90° with no rotation of the wrist. Participants were instructed to squeeze the dynamometer as tightly as possible for a period of five seconds. The result was recorded, and the test repeated three times on each hand. Participants carried out the test in the right hand first. Between each repetition a minimum of 30 seconds rest was given. The maximal grip strength from the six measurements was used for analysis.

2.17 Biochemistry

A summary table of biochemical measurements and corresponding measurement method is shown in **table 6**. A summary of biochemical measurements by time point can be seen in **table 7**.

2.17.1 Sample collection

Non-fasting blood samples were collected at visit 1 to confirm eligibility. Screening biochemical analysis was performed by the Clinical Chemistry Laboratory (Sheffield Teaching Hospitals, UK). Participants were screened for total 25(OH)D. To help interpret vitamin D results, creatinine, phosphate, calcium and albumin were also measured.

Blood samples were collected at each remaining time point, after an overnight fast. Samples were collected between 8.00am and 11.00am to minimize the effect of circadian rhythms and inter-individual variability on circulating concentrations of measured analytes.

2.18.2 Sample handling

Blood samples were collected for measurement of vitamin D metabolites, bone turnover markers and other biochemical factors of interest. Samples were collected into Serum Separating Tubes (SST) and EDTA vacutainer tubes. Blood in SST tubes was left to clot for 30 minutes at room temperature and centrifuged at 3000rpm for 10 minutes. The serum was aliquoted and stored at -80°C until analysis. For plasma, blood samples were collected into EDTA tubes and were centrifuged at 3000rpm for 15 minutes. The plasma was aliquoted and stored at -80°C until analysis.

2.19.3 Assay principles & Biochemical measurements

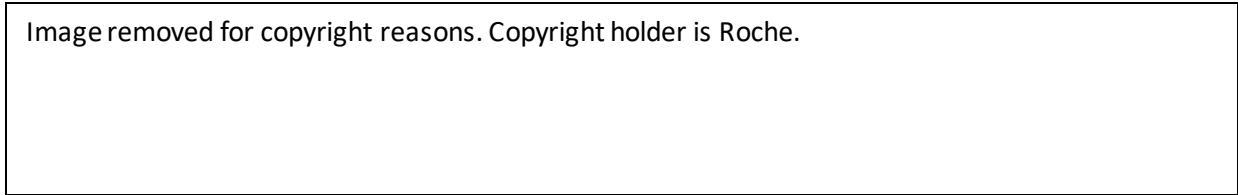
Total 25(OH)D measured by ElectroChemiLuminescence ImmunoAssay (ECLIA)

Total 25(OH)D was measured at screening using a Cobas e411 autoanalyser (Roche Diagnostics, Mannheim, Germany), which uses an ECLIA to detect small analyte concentrations. The total 25(OH)D competitive assay uses VDBP as a capture protein to bind 25(OH)D₂ and 25(OH)D₃ in a sample (**figures 20 & 21**).

During the first incubation step with a pre-treatment reagent, bound 25(OH)D is released from VDBP in the sample. Ruthenium labelled VDBP is added to the sample and during a second incubation step a complex is formed between the 25(OH)D in the sample and the ruthenylated VDBP. Streptavidin-coated microparticles and biotinylated 25(OH)D are added for a third incubation step and the unbound ruthenium labelled VDBP become occupied. A complex of ruthenylated VDBP and biotin labelled 25(OH)D is formed. The entire complex is bound to the microparticles via the interaction between biotin and streptavidin. The mixture is aspirated into a measuring cell. The microparticles are magnetically captured to the surface of the measuring cell. A reagent is added to wash away unbound particles and to provide Tripropylamine (TPA). Ruthenium and TPA are highly stable at base state and only when voltage is applied do the reactants begin emitting photons. The ruthenium and TPA are oxidized

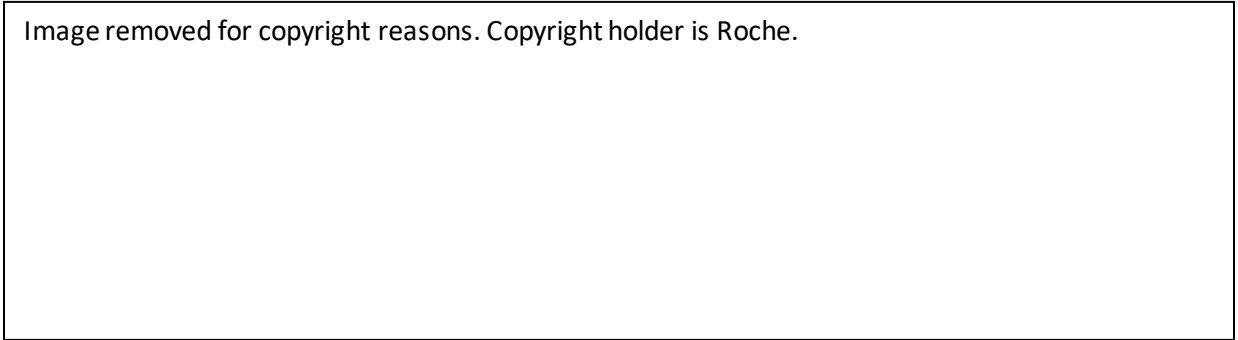
upon application of a specific voltage to an electrode. The TPA releases a proton which reacts with ruthenium, reducing it to an excited state. As the ruthenium decays from its excited state to its lower energy basal state, light is emitted. The light emission is measured by a photomultiplier. The amount of light produced is quantified against a calibration curve and is indirectly proportional to the amount of 25(OH)D in the sample. The manufacturers inter-assay CV is reported at <5.5%.

Figure 20: *Principles of a competitive protein binding assay.*



Source: *Roche Diagnostics vitamin D total assay factsheet (2012)*

Figure 21: *Generation of luminescence in Electrochemiluminescence (ECLIA).*



Source: *Roche Diagnostics (2011)*

Total 25(OH)D measurement post-screening

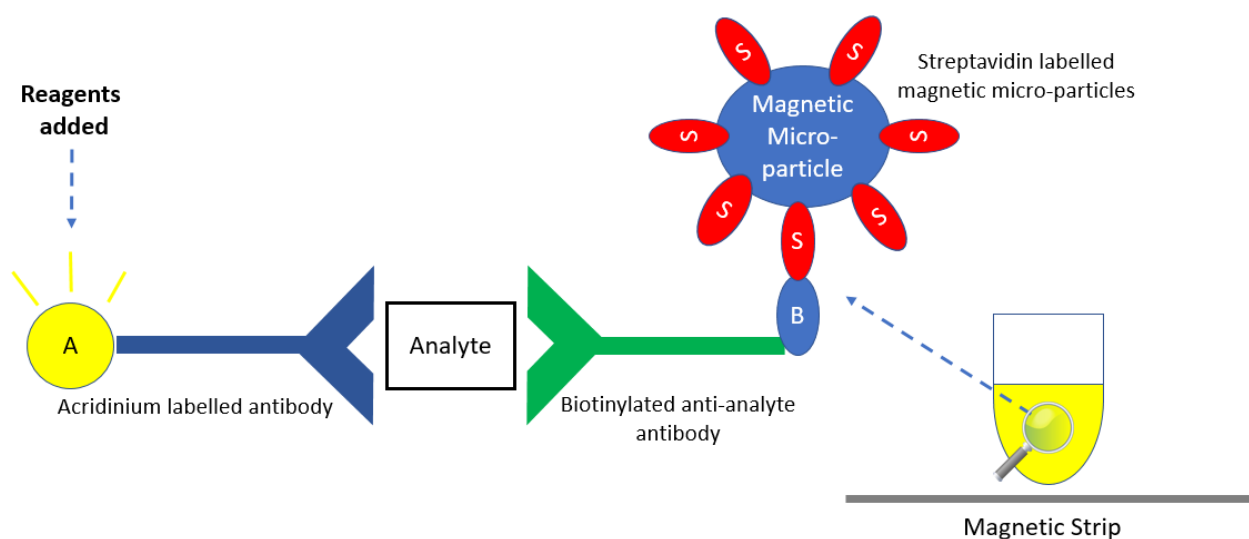
At baseline and subsequent time points, total 25(OH)D was measured by CLIA (ImmunodiagnosticSystems, Boldon, UK) at the Bone Biochemistry Laboratory (University of Sheffield, UK). The inter-assay CV was 5.7%.

Principles of a Sandwich Chemiluminescence ImmunoAssay (CLIA)

The IDS-iSYS (ImmunodiagnosticSystems, Boldon, UK) uses CLIA to detect and quantify sample analytes. This technique uses two antibodies that detect and bind different portions of the analyte molecule, resulting in the formation of a sandwich complex.

The appropriately diluted serum or plasma sample is loaded onto the autoanalyzer. A biotinylated anti-analyte antibody and an acridinium labeled antibody are added. Streptavidin coated magnetic micro-particles are added and these bind to the biotin in the complex. After incubation, the magnetic micro-particles are captured using a magnetic strip and any unbound analyte is removed during a wash step. Trigger reagents are added to stimulate the acridinium conjugate to emit light. The amount of light emitted is directly proportional to the concentration of the analyte in the sample. The principles of the assay are illustrated in **figure 22**.

Figure 22: Principles of chemiluminescence immunoassay (CLIA).



Source: Self-drawn by author

25(OH)D₂ & 25(OH)D₃

Immunoassays may fail to discriminate between 25(OH)D₂ and 25(OH)D₃ and do not recognize 25(OH)D₂ as well as 25(OH)D₃ (156, 158). Therefore, total 25(OH)D₂ and 25(OH)D₃ were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the laboratory of the Institute of Human Development (University of Manchester, UK). This is currently the gold standard for measuring total 25(OH)D (131). The LC-MS/MS is also the method used by the National Institute of Standards and Technology (158). An overview of the method is as follows; 200 μ L samples and a deuterated internal standard (d6-25(OH)D) were prepared using 100 μ L methanol;

isopropanol (80:20) and then extracted with 1mL of hexane. This extracted 25(OH)D was blown down, reconstituted in 150µL of 66% methanol and injected onto a Waters Phenyl Column attached to the mass spectrometer. The extract was eluted with an isocratic gradient over 5 minutes. Analysis was carried out in positive ion mode using the transitions m/z 401>159 for 25(OH)D and Mm/z 407>159 for d6-25(OH)D.

1,25(OH)₂D

Total 1,25(OH)₂D by was measured by CLIA after an extraction step on the IDS-iSYS (ImmunodiagnosticSystems, Boldon, UK). The inter-assay CV was 6.0%.

PTH

Intact PTH measured by an automated sandwich CLIA on the IDS-iSYS (ImmunodiagnosticSystems, Boldon, UK). Two polyclonal antibodies for PTH were used; a biotin-labelled antibody that recognizes amino acids 39-84 (C-terminal region) was used as the capture antibody and a acridinium-labelled antibody that recognizes amino acids 13-34 (the N-terminal region) was used as the detection body. The manufacturers inter-assay CV is <2.0%.

Manual Immunoassay (ELISA)

Immunoassays detect a label measure to measure the amount of analyte (antigen) or antibody which is present in a sample. Manual immunoassays were used to measure free 25(OH)D and VDBP. The manual immunoassays used in this study can be classified as competitive and non-competitive.

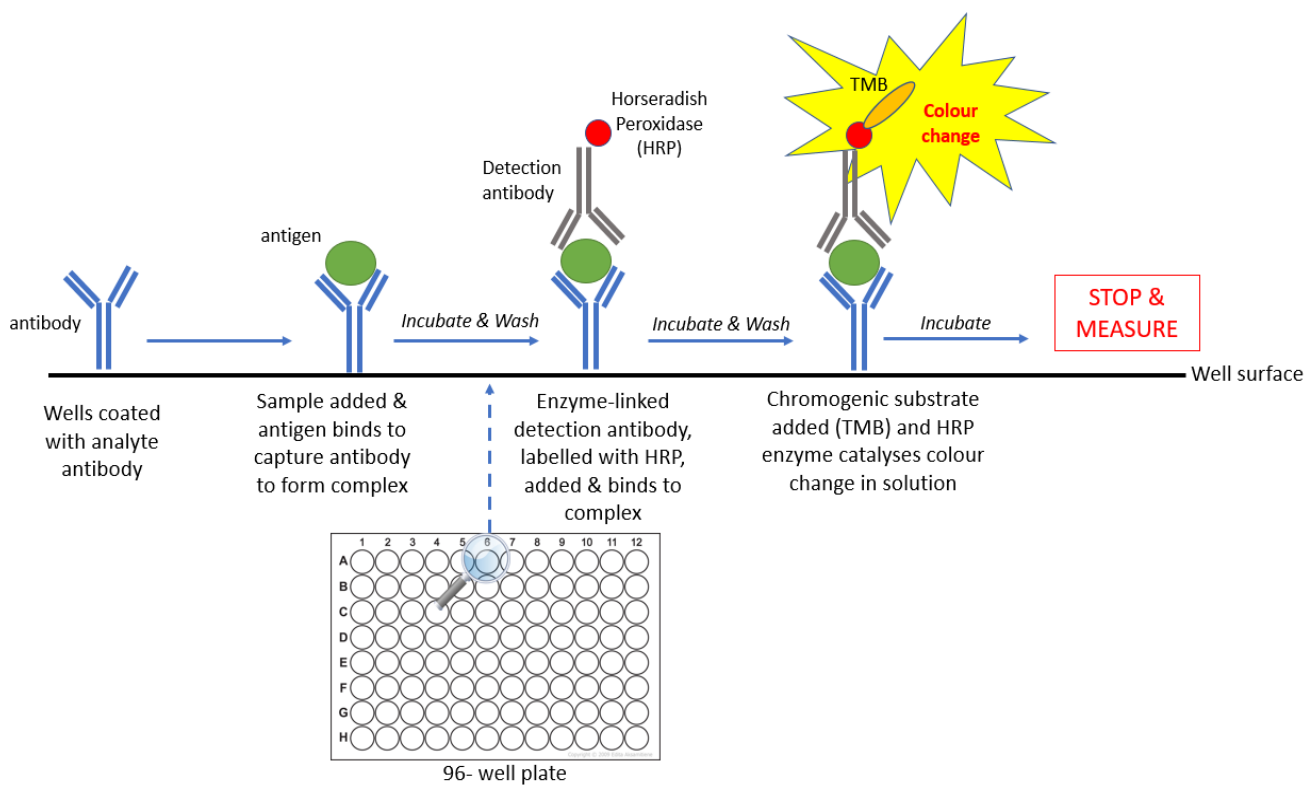
Vitamin D Binding Protein

VDBP was measured using a non-competitive two-site enzyme-linked Sandwich immunoassay (Genways, San Diego, USA) at the Bone Biochemistry Laboratory (University of Sheffield, UK). This assay uses polyclonal antibodies to bind the VDBP. VDBP can be measured using monoclonal or polyclonal antibodies. However, assays that use polyclonal antibodies appear to be less subject to variations in the structure of VDBP that occur due to polymorphisms in the VDBP (158). Previously, VDBP concentrations have been reported to differ between populations groups with different VDBP alleles when measured using a monoclonal antibody (151). However, more recently, when measured using the polyclonal antibody-based assays there is no difference in VDBP concentration across different allelic sub-groups (165, 171).

The principles of the assay are illustrated in **figure 23**. Serum samples (1/40 000) and standards were diluted and added to the 96-well microplates that are pre-coated with anti-VDBP antibodies. The anti-VDBP antibodies have been absorbed to the surface of the polystyrene microtiter wells and any VDBP present in the sample binds to the

antibody. Following an incubation period, all unbound proteins were removed by washing. An Anti-VDBP antibody conjugated with horseradish peroxidase (HRP) was then added and form complexes with the bound VDBP. After another incubation period and washing step, a chromogenic substrate (3,3',5,5'-tetramethylbenzidine (TMB)) was added and the plate kept in darkness. TMB acts as a hydrogen donor in the reduction of hydrogen peroxidase to water by HRP and this causes the solution to take on a blue colour. After a further incubation step (10 minutes) an acidic stop solution was added and the colour changed to yellow. The quantity of the bound enzyme varies directly with the concentration of VDBP in the tested sample and therefore the density of yellow coloration read by absorbance at 450 nm on a microplate reader is quantitatively proportional to the amount of VDBP in the tested sample. The quantity of VDBP can be interpolated from the standard curve constructed from the standards and corrected for sample dilution. The inter- and intra- assay CV were 3.3% and 3.9%, respectively.

Figure 23: Principles of a sandwich immunoassay (ELISA)



Source: Self-drawn by author

Free 25(OH)D

Calculated Free 25OHD

Free 25(OH)D was calculated from the using the concentrations of albumin and VDBP and their respective binding affinities for 25(OH)D. The formula used was (153):

$$\text{Free 25(OH)D} = \text{Total 25(OH)D} / (1 + (6 \cdot 10^5 \times \text{albumin}) + (7 \cdot 10^8 \times \text{DBP}))$$

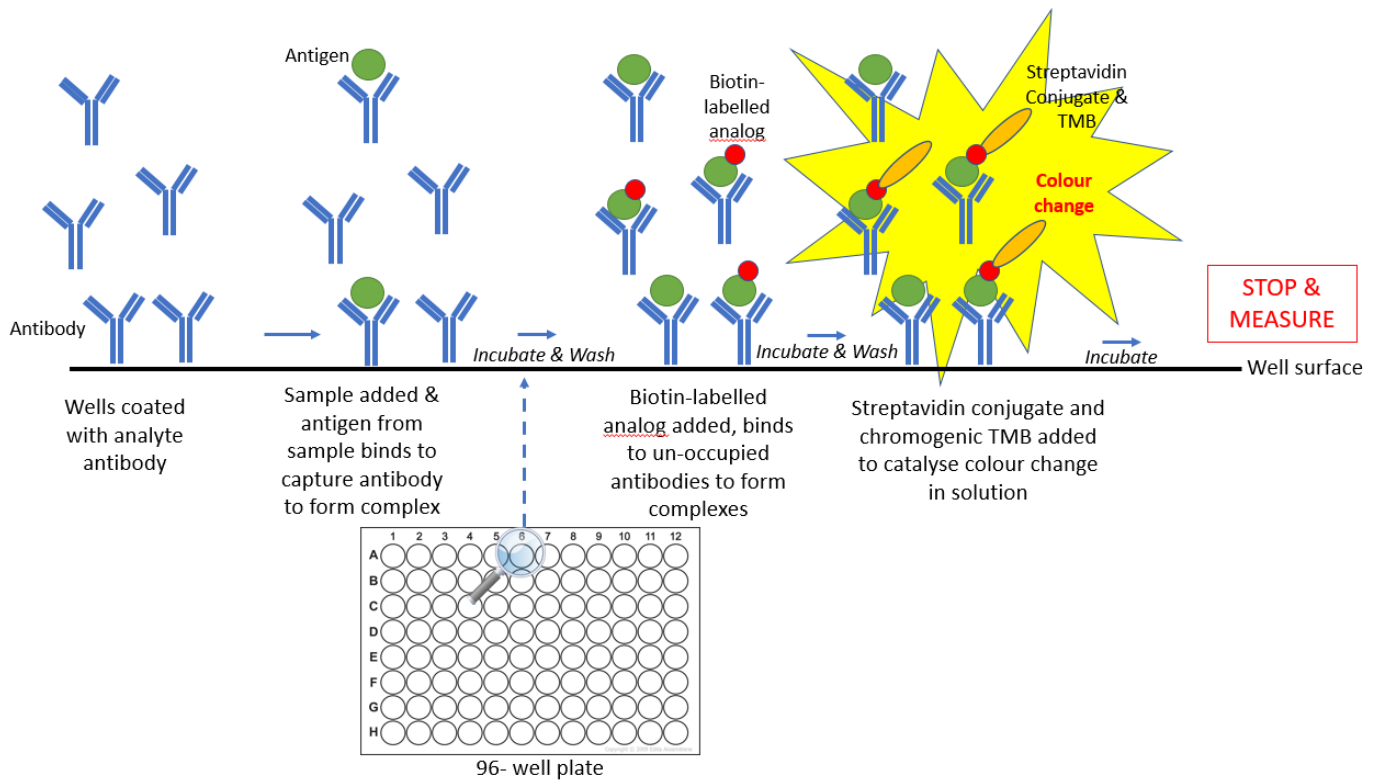
Despite recent advancements in our understanding that VDBP concentration does not seem to be different across different VDBP alleles (165), there is still the issue of binding affinity to consider. In using this calculated method to estimate the free fraction of 25(OH)D there is the assumption that all alleles have the same binding affinity for different vitamin D metabolites, but this may not be the case as differences in the rank order for binding affinity across alleles has been demonstrated (156).

Directly Measured Free 25(OH)D

Due to limitations with the calculated method, free 25(OH)D was also measured by a manual competitive immunoassay (Future Diagnostics BV, Wijchen, Netherlands) at the Bone Biochemistry Laboratory (University of Sheffield, UK). Competitive immunoassays are based on the competition for the anti-analyte antibody between the analyte in the tested sample and a labelled analyte.

Serum samples and calibrators were pipetted into the wells of a microtiter plate coated with anti-25(OH)D antibody. During the first incubation step, the free 25(OH)D is captured by the antibody. The solid phase was then washed and a biotinylated analog of 25(OH)D was added and allowed to bind with the non-occupied antibody binding sites during a second incubation phase. Following a second wash, and incubation with a streptavidin peroxidase conjugate, a TMB chromogenic substrate was added. A stop reagent was added, and the bound enzyme is quantified using a colorimetric reaction. The absorbance (450nm) was measured on a microplate reader. The concentration of free 25(OH)D in the tested sample is inversely proportional to the absorbance in the sample well. The inter- and intra- assay CV were 5.8% and 2.6%, respectively. The principles of the assay are illustrated in **figure 24**.

Figure 24: Principles of a manual competitive immunoassay



Source: Self-drawn by author

Calculated Free 1,25(OH)₂D

Free 1,25(OH)₂D was also calculated from the using the concentrations of albumin and VDBP and their respective binding affinities for 1,25(OH)₂D. The formula used was (152):

$$\text{Free } 1,25(\text{OH})_2\text{D} = \text{Total } 1,25(\text{OH})_2\text{D} / (1 + (5.4 \times 10^4 \text{M}^{-1} \times \text{albumin}) + (3.7 \times 10^7 \text{M}^{-1} \times \text{DBP}))$$

Bone Turnover Markers

Bone is a dynamic tissue which undergoes constant remodelling throughout life. Markers of bone resorption and bone formation are novel tools which detect the dynamics of bone remodelling (297). Changes BTMs reflect acute changes to bone that may not be reflected in bone imaging. The currently available markers of bone turnover include enzymes and non-enzymatic peptides that are derived from the cellular and non-cellular compartments of bone (297). Bone formation markers reflect different stages of osteoblast formation and different aspects of osteoblast function (298) (**figure 25**). They are categorized as by-products of collagen synthesis (e.g. pro-peptides

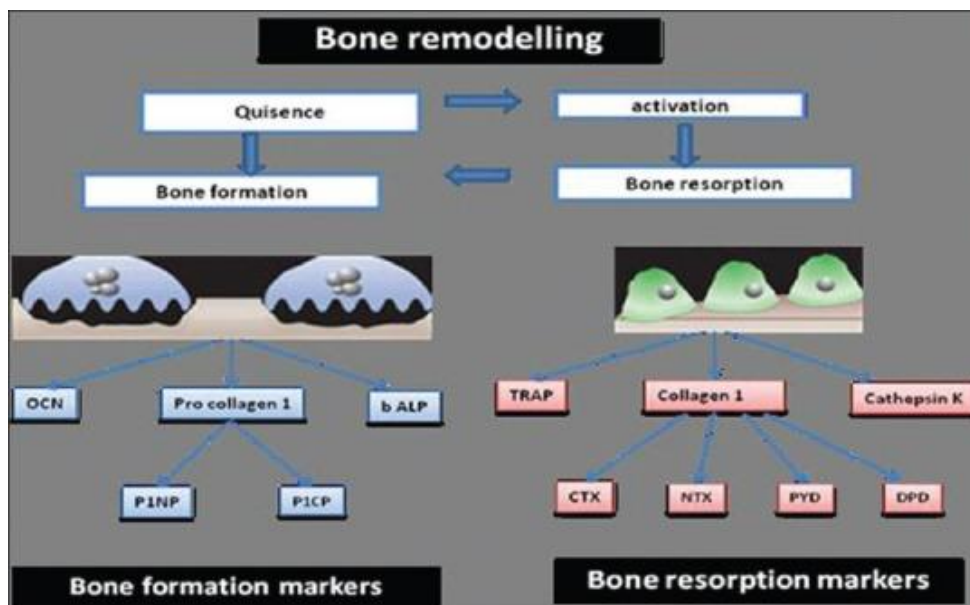
of type-1 collagen), osteoblast enzymes (e.g. alkaline phosphatase) or matrix proteins (e.g. osteocalcin). Bone resorption markers are formed during the resorption phase of bone remodelling and include by-products of osteoclastic activity. Bone resorption markers are categorised as collagen degradation products (e.g. CTX), non-collagenous proteins, osteoclastic enzymes (e.g. tartrate-resistant acid phosphatase and cathepsin K) or osteocyte activity markers (e.g. sclerostin, dickkopf-related protein-1 and osteoprotegerin).

Serum CTX and PINP were measured in line with recommendations of the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine Working Group on Bone Marker Standards (IOF/ICC WG-BMS) (299).

Osteoblasts secrete procollagen (a precursor of type 1 collagen) during bone formation. Peptides at each end of the procollagen molecule (procollagen type 1 N propeptide and procollagen type 1 C propeptide) are cleaved by enzymes during the bone formation process and are released into circulation. PINP is chosen as the reference bone turnover markers because it is the most abundant protein of bone tissue and most PINP is believed to be derived from bone (during bone formation). It is also widely used for fracture prediction and monitoring of osteoporosis treatment and there is good assay precision and stability during sample handling (297, 298).

Osteocalcin (OC) is an abundant non-collagenous protein in the bone matrix and is exclusively synthesized by osteoblasts during bone formation. OC was measured as an addition bone formation marker.

Figure 25: Summary of bone turnover markers



Source: Shetty et al., (297)

CTX are degradation products of type 1 collagen and are measured as bone resorption markers. CTX is generated by the osteoclastic hydrolysis of collagen, generated by cathepsin K. CTX exists in circulation two isomerized forms; α and β . β CTX is a marker of the degradation of mature collagen and has been measured in this study, whereas α CTX is a marker of the degradation of immature collagen. Despite, serum CTX being acknowledged as the reference marker for bone resorption by the IOF/ICC WG-BMS, consideration is still needed for analytical and preanalytical variability. Serum CTX measurements are influenced by renal function, liver function and by circadian variation, with peak levels recorded at 5.00am and lowest levels seen at 2.00pm (300). Serum CTX is also lower post-prandial by up to 20% compared to a fasted state (301). **Table 8** explains how these sources of variability were controlled for.

PINP, OC and CTX-I were measured by an automated sandwich CLIA on the IDS-iSYS (Immunodiagnostic Systems, Boldon, UK).

For PINP, two monoclonal antibodies were used. For OC, two highly specific monoclonal antibodies were used. The capture antibody labelled with biotin recognises the mid-region (amino acids 20-29) and the detection antibody labelled with acridinium recognises the N-terminal region (amino acids 10-16). Two highly specific monoclonal antibodies for the amino acid sequence EKAHD- β -GGR were used to measure CTX-I. The inter-assay CV for PINP, OC and CTX was 5.1%, 2.6% and 2.8%, respectively.

Other Biochemistry

To help interpret vitamin D and PTH results accurately, creatinine, calcium, albumin and phosphate were measured at each time point. Blood lipids were also measured at baseline as these are potential confounders of vitamin D measurements.

Serum calcium and 24-hour urine calcium

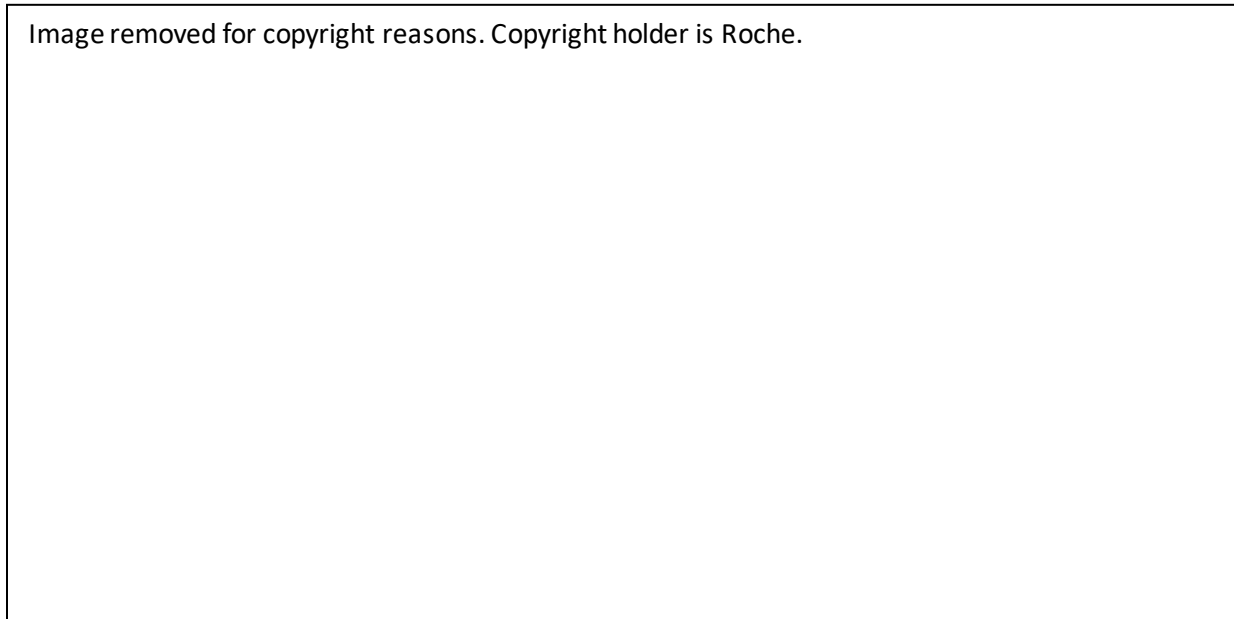
Serum calcium and 24-hour urine calcium were measured using an automated colorimetric assay on the Cobas c701 (Roche Diagnostics, Mannheim, Germany) in the Chemical Chemistry laboratory (Sheffield Teaching Hospitals, UK).

A reagent is added to the sample and calcium ions in the sample bind with the 5-nitro-5' methyl-BAPTA (NM-BAPTA) indicator during an incubation phase. A calcium-NM-BAPTA complex forms (One calcium molecule binds to one NM-BAPTA molecule) and this leads to a colour change which is measured photometrically. During a second step, EDTA is added and reacts with the calcium-NM-BAPTA complex. The calcium ion is snatched from the calcium-NM-BAPTA complex by the EDTA due to a higher binding affinity. A calcium-EDTA complex is formed and NM-BAPTA is released. This causes a change in absorbance which is directly proportional to the calcium concentration in the original

sample. The manufacturer's reported inter assay precision was <2.0%. A summary of the principles of the assay can be seen in **figure 26**.

Serum ionised calcium was measured using a ABL90 Flex analyser (Radiometer, Denmark). The manufacturer's reported inter assay precision was <2.0%.

Figure 26: Principles of the photometric assay used to determine serum and urinary calcium



Source: Roche Diagnostics (2012)

Serum and urinary creatinine

Serum and 24-hour urine creatinine was measured by colorimetric assay on the Cobas c701 automated analyser (Roche Diagnostics, Mannheim, Germany) in the Chemical Chemistry laboratory (Sheffield Teaching Hospitals, UK). The assay is based on the Jaffe Method. In brief, creatinine forms a yellow-orange complex with picric acid in alkaline solution. The rate of colour change is proportional to the creatinine concentration in the sample. The manufacturer's reported inter assay precision was <2.0%.

Serum phosphate

Serum phosphate was measured by colorimetric assay on the Cobas c701 automated analyser (Roche Diagnostics, Mannheim, Germany) in the Chemical Chemistry laboratory (Sheffield Teaching Hospitals, UK). The assay principle

is that in the presence of sulphuric acid, inorganic phosphate forms an ammonium phosphomolybdate complex with ammonium molybdate. The concentration of phosphomolybdate formed is directly proportional to the phosphate concentration in the sample and is measured photometrically. The manufacturer's reported inter assay precision was <2.0%.

Albumin

Serum albumin was measured by colorimetric assay on the Cobas c701 automated analyser (Roche Diagnostics, Mannheim, Germany) in the Clinical Chemistry laboratory (Sheffield Teaching Hospitals, UK).

The assay principle is that at a pH of ~4.1, albumin is able to bind with bromocresol green (BCG) to form an albumin-BCG complex. This causes a colour change in the indicator from yellow to blue/green. The colour intensity is directly proportional to the albumin concentration in the sample and is measured photometrically. The manufacturer's reported inter assay precision was <2.0%.

Lipids

Triglycerides, Total Cholesterol and HDL were also measured by automated ECLIA (Cobas c701, Roche Diagnostics, Mannheim, Germany) in the Chemical Chemistry laboratory (Sheffield Teaching Hospitals, UK). The manufacturer's reported inter assay precision was <2.0% for each test.

Low density lipoprotein (LDL) was calculated using the Friedwald Equation:

$$\text{LDL} = \text{Total cholesterol (mmol/l)} - \text{HDL cholesterol (mmol/l)} - (\text{triglycerides (nmol/l)} / 2.2)$$

Renin and aldosterone

Renin and aldosterone were measured by LC-MS/MS at the University of Manchester Institute of Human Development. The inter assay CV was 6.6% and 7.8%, respectively. The aldosterone to renin ratio (ARR) was calculated by dividing the concentration of aldosterone by the renin activity.

Table 6: Summary of biochemical measurements

Analyte/Outcome	Site of Measurement	Method of Measurement	Sample
Free 25(OH)D	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	Immunoassay	Serum
Real time 25(OH)D (if required for eligibility confirmation)	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	ECLIA	Serum
25(OH)D (D2 and D3)	University of Manchester (Institute of Human Development) & Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	LC-MS/MS iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	Serum
1,25(OH) ₂ D	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	Serum
Vitamin D Binding Protein	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	Manual Immunoassay (R & D Systems, UK)	serum
PTH	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	serum
Creatinine	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	serum
Ionized Calcium	Cystic Fibrosis Unit, Sheffield Teaching Hospitals UK	Gas Analyser (Radiometer, Denmark)	whole blood
Calcium	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	serum
Phosphate	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	serum
Albumin	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	serum
Lipids (Cholesterol, LDL, HDL & triglycerides)	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	Serum
24h urine Ca Cr	Chemical Pathology Laboratory, Sheffield Teaching Hospitals, UK	Cobas e411 automated immunoassay analyser (Roache Diagnostics, Germany)	24h urine
B Carboxy-terminal collagen crosslinks	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	serum
Procollagen 1 N-terminal propeptide	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	serum
Osteocalcin	Bone Biochemistry Laboratory (Academic Unit of Bone Metabolism, University of Sheffield)	iSYS IDS automated immunoassay (ImmunoDiagnostic Systems, UK)	serum
Renin	University of Manchester (Institute of Human Development)	LC-MS/MS	Plasma
Aldosterone	University of Manchester (Institute of Human Development)	LC-MS/MS	Plasma

Table 7: Summary of biochemical measurements by study visit

Study Visit	V1	V2	V3*	V4*	V5
Free 25(OH)D (Serum)		X	X	X	X
Real time 25(OH)D	X*				
25(OH)D (D2 and D3)		X	X	X	X
1,25(OH) ₂ D		X	X	X	X
Vitamin D Binding Protein		X	X	X	X
PTH		X	X	X	X
Creatinine		X	X	X	X
Ionized Calcium		X	X	X	X
Calcium		X	X	X	X
Phosphate		X	X	X	X
Albumin		X	X	X	X
Lipids (Cholesterol, LDL, HDL & triglycerides)		X			
24h urine Ca Cr		X	X	X	X
CTX		X	X	X	X
PINP		X	X	X	X
OC		X	X	X	X
Renin & Aldosterone		X	X	X	X

*Treatment group only

2.16.4 Biochemistry Quality Control

The Bone Biochemistry Laboratory (University of Sheffield) and Manchester Institute of Human Development (MIHD) take part in The Vitamin D External Quality Assurance Scheme (DEQAS) which serves as a quarterly monitor of performance of analysts and 25(OH)D analytical methods across laboratories worldwide (302). The 25(OH)D₃ assay completed at the MIHD was also calibrated against the National Institute of Standards and Technology (NIST) reference standards, using a validated LC-MS/MS method. These quality assurance schemes help to limit inter-laboratory assay-specific differences in this status marker.

To minimise technician variability, all assays were carried out by, or under the supervision of, an experienced laboratory technician and samples were analysed in duplicate for manual assays. All samples underwent a maximum of two freeze-thaw cycle. A fresh sample was used where repeated freeze-thaw cycles were indicated as unsuitable according to the manufacturer's guidelines.

The type of analyte measurement method used affects variability. Manual ELISAs are subject to more operator error than automated assays. ECLIA and CLIA are highly sensitive, allow for a large measurement range (reducing the need for dilution and repeats), they have short incubation times and allow for fast throughput and use non-isotopic labels (303). The use of monoclonal antibodies in most automated methods used in this study, also improves specificity for the given analyte. Analyte measurements are also affected by pre-analyte variability. The sources of pre-analytical variability and measures used to modify these elements of variability are summarised in **table 8**.

Table 8: Factors affecting pre-analytical variability and measures put in place to control for this variability

	Source of Variability	Measures to control variability
Modifiable	Circadian rhythm	All samples collected between 8.00am and 10.00am
	Menstrual variation	All participants >5 years postmenopausal
	Fasting/food intake	All study visit (after screening) taken after overnight fast
	Physical activity	Participants instructed to not vigorously exercise for 24 hours prior to sample collection
	Seasonal variation	All samples collected during the winter months
Less modifiable	Age	All participants over the age of 55 years
	Sex	All participants female
	Menopausal status	All participants older women > 5 years postmenopausal
	High bone turnover diseases	e.g. Primary hyperparathyroidism, bone metastasis, Paget's disease excluded
	Low bone turnover diseases	e.g. hypothyroidism, hypoparathyroidism excluded
	Diseases characterised by disassociation between bone formation and bone resorption	e.g. Cushing's disease and multiple myeloma
	Chronic diseases associated with low mobility	Participants ineligible/excluded
	Recent fracture	Participants with fracture >12-months prior recruitment ineligible/excluded
	Medications known to affect bone turnover	e.g. corticosteroids, aromatase inhibitors, antiepileptics; Participants taking such medication ineligible/excluded
	Day-to-day variation	not controlled for

2.17: Statistical analysis and presentation of baseline data (Chapter 3)

Each variable is presented as median and inter-quartile range (IQR) for each treatment group and the control group. For comparisons of baseline variables for all treatment groups combined with the control group, each variable was tested for normality with a Shapiro-Wilk test. Normality was assumed if Shapiro-Wilk test was not statistically significant ($P > .05$). Where data were normally distributed, the data are presented as mean and standard deviation (SD). Where data were not normally distributed, the data are presented as median and IQR. An independent samples t-test was used to determine between-group differences where data in both groups (treatment groups combined vs. control group) were normally distributed. Levene's test was used to determine homogeneity of variances between the comparison groups. Equality of variances was assumed where $P > .05$. A Mann-Whitney U test was used to test for between-group differences where data were not normally distributed in one or both of the comparison groups.

2.18 Inferential statistical analysis (Chapters 4 & 5):

Variables that did not meet the assumptions of normality were log transformed (\log_{10}) before inferential analysis and are expressed as geometric means and 95% confidence intervals. Where variables were not log transformed these are expressed as the arithmetic mean and 95% confidence intervals.

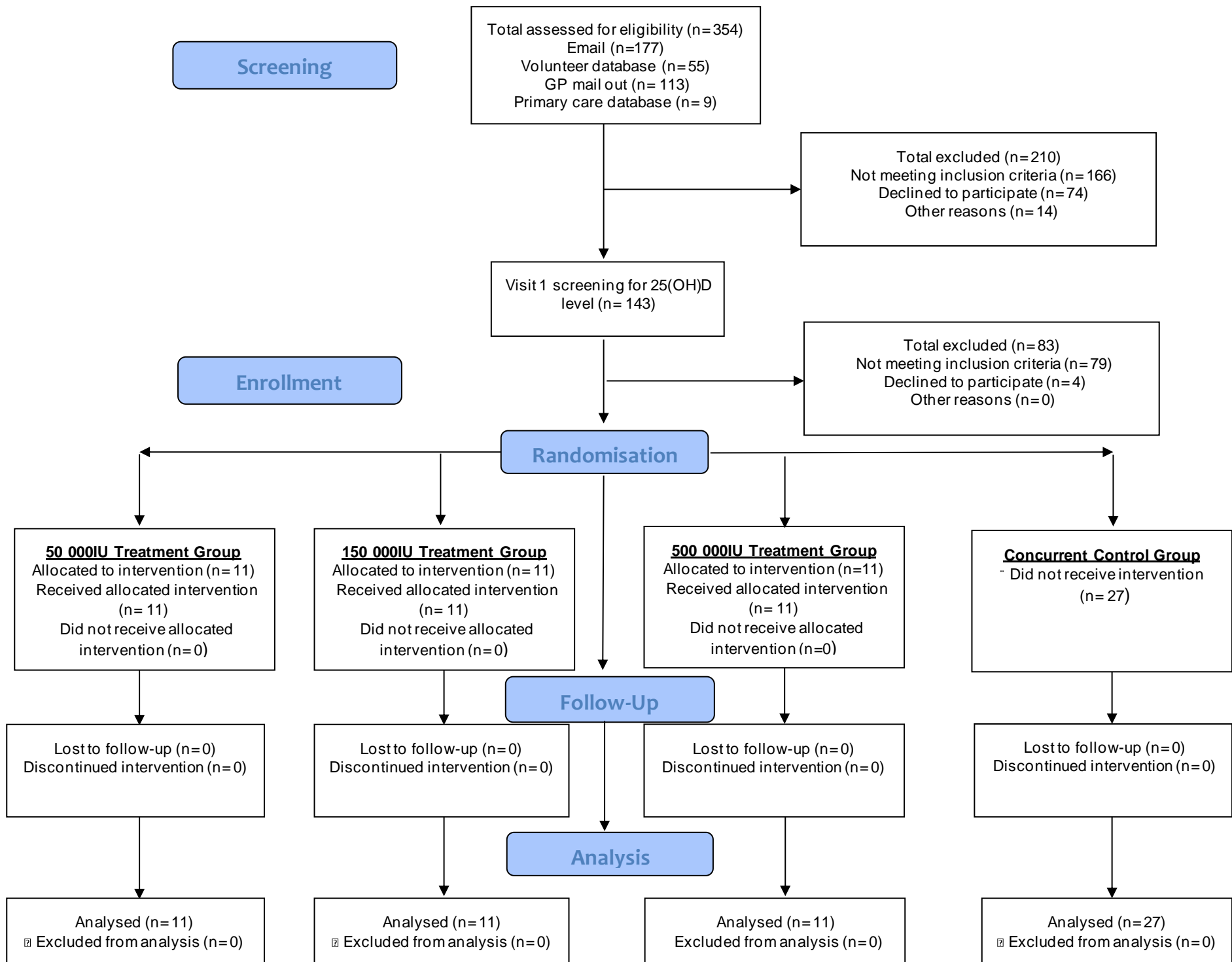
A mixed effect model was used (with treatment group as a between factor and time as interaction factor) to assess whether there were any differences between treatment groups and whether the any differences changed over time. A significant group x time interaction effect ($P < .05$) indicated that the within-group changes over time were significantly different between treatment groups. Where there was a significant interaction between group and time, post hoc pairwise contrasts (Tukey test) were programmed to determine where the differences existed (at specific time points between the treatment groups). Where there was no significant interaction effect, a main effect of treatment was indicated where $P < .05$. Proportional within-group changes over time are reported as a ratio (95% CIs) of baseline levels where variables have been log transformed. Proportional within-group changes are reported as an absolute mean difference (95% CIs) where variables have not been log transformed.

A one-way ANOVA was used to compare treatment groups at week 12. Where the ANOVA was $P < 0.05$, post-hoc analysis was carried out to determine specific between-group differences.

Control group comparison between baseline and week-12 was done using a related measures (paired samples) t-test. All tests were performed with a $P < .05$ significance level.

Chapter 3:

Baseline Characteristics



3.0 Baseline characteristics

Table 9 shows baseline demographics, dietary vitamin D and calcium intake and sunlight exposure scores in each treatment group and the control group. **Table 10** shows baseline biochemical parameters for each treatment group and the control group and **table 11** shows baseline physical function scores and cardiovascular parameters for each group.

Between-group comparisons of baseline demographics, dietary intake and sunlight exposure scores for all treatment groups combined and the control group are shown in **table 12**. Between-group comparisons of baseline biochemical parameters for treatment groups combined and the control group are shown in **table 13**. Between-group comparisons of baseline physical function scores and cardiovascular measures for treatment groups combined and the control group are shown in **table 14**.

Table 9: Baseline demographics, dietary intake and sunlight exposure by treatment group.

	50 000IU	150 000IU	500 000IU	Control
	(n=11)	(n=11)	(n=11)	(n=27)
Age (Years)	63	62	62	60
	(57-66)	(58-69)	(55-68)	(58-64)
Height (cm)	163	157.2	159.9	160.0
	(157.5-166.7)	(156.3-163.3)	(156.6-163.6)	(155-165.5)
Weight (kg)	65.5	64.3	63.6	66.1
	(60.8-74.0)	(57.1-64.3)	59.3-67.0	(62.4-72.0)
BMI (kg/m ²)	24.6	23.2	23.6	26.4
	(22.7-28.5)	(22.6-28.1)	(23.2-27.6)	(23.0-28.7)
Total dietary vitamin D intake (µg/day)	1.6	0.87	2.6	2.2
	(1.4-4.0)	(0.7-1.1)	(1.5-2.6)	(0.9-3.2)
Total dietary calcium intake (mg/day)	677	637	1013	806
	(649-1048)	(436-800)	(737-1071)	(668-981)
Sunlight exposure score	83	83	59	80
	(72-122)	(42-88)	(34-108)	(54-110)

All values are median (IQR)

Table 10: Baseline biochemical parameters by treatment group.

	50 000IU	150 000IU	500 000IU	Control
	(n=11)	(n=11)	(n=11)	(n=27)
Total 25(OH)D (nmol/l)	30.6 (27.8-34.5)	33.2 (26.1-42.1)	30.9 (27.8-35.6)	67.1 (53.7-86.8)
Total 25(OH)D ₃ (nmol/l)	20.2 (17.1-23.4)	19.5 (18.1-23.6)	20.4 (16.9-27.1)	54.8 (44.1-67.9)
Measured free 25(OH)D (pmol/l)	6.2 (5.6-6.9)	7.0 (5.9-8.2)	6.0 (4.1-7.0)	13.8 (9.9-15-2)
Calculated free 25(OH)D (pmol/l)	8.1 (5.9-8.5)	7.8 (7.0-11.2)	7.0 (6.1-10.1)	15.7 (12.3-20.6)
Calculated free 25(OH)D ₃ (pmol/l)	4.6 (4.0-6.4)	4.6 (4.2-6.0)	4.9 (4.0-7.7)	14.3 (10.5-17.0)
Total 1, 25(OH) ₂ D (pmol/l)	60.5 (44.3-78.0)	63.3 (53.0-70.7)	72.8 (50.0-92.8)	81.0 (63.0-105.5)
Calculated free 1, 25(OH) ₂ D (fmol/l)	240 (208-315)	261 (210-312)	280 (210-446)	385 (253-456)
PTH (pg/ml)	40.3 (28.0-51.4)	39.4 (33.8-46.6)	37.8 (30.9-47.4)	34.7 (24.2-40)
Serum calcium (mmol/l)	2.32 (2.30-2.35)	2.35 (2.32-2.41)	2.30 (2.24-2.33)	2.32 (2.26-2.40)
Ionized calcium (mmol/l)	1.24 (1.21-1.27)	1.24 (1.23-1.28)	1.24 (1.22-1.27)	1.24 (1.23-1.28)
Serum Phosphate (mmol/l)	1.17 (1.09-1.27)	1.25 (1.13-1.32)	1.20 (1.04-1.27)	1.18 (1.07-1.24)
Serum Creatinine (μmol/l)	64 (58-76)	67 (62-69)	66 (59-72)	65 (59-76)
24hr urine Ca (mmol/24hrs)	2.4 (1.3-3.5)	2.3 (1.5-2.6)	3.4 (2.6-5.0)	2.8 (2.0-4.8)
24hr urine Cr (mmol/24 hrs)	8.0 (6.8-8.9)	8.2 (5.2-8.7)	8.1 (7.0-10.1)	9.0 (7.3-10.5)

Table 10 (cont): Baseline biochemical parameters by treatment group.

	50 000IU	150 000IU	500 000IU	Control
	(n=11)	(n=11)	(n=11)	(n=27)
24hr urine Ca:Cr	0.31 (0.16-0.46)	0.29 (0.24-0.34)	0.47 (0.33-0.60)	0.31 (0.22-0.48)
FGF-23 (pg/ml)	42.6 (31.5-46.4)	45.8 (42.6-75.6)	44.9 (39.7-60.9)	43.1 (33.6-47.3)
Albumin (g/L)	47 (47-49)	47 (46-48)	48 (46-49)	47 (45-49)
VDBP (µg/ml)	290 (256-359)	313 (260-336)	305 (262-343)	299 (265-340)
PINP (ng/ml)	51.9 (48.6-68.0)	51.0 (43.9-64.5)	53.4 (40.1-70.3)	57.2 (47.4-66.8)
Osteocalcin (ng/ml)	21.8 (20.4-30.1)	25.1 (22.4-25.5)	26.1 (20.5-36.6)	24.9 (21.4-32.3)
CTX (ng/ml)	0.44 (0.33-0.55)	0.51 (0.41-0.68)	0.55 (0.31-0.74)	0.49 (0.32-0.58)
Renin (ng/ml//hr)	2.1 (1.3-4.2)	2.5 (2.1-3.0)	1.7 (1.3-3.4)	2.5 (1.7-4.2)
Aldosterone (ng/dl)	7.8 (5.4-14.1)	8.0 (4.4-10.5)	7.6 (5.0-9.5)	7.5 (5.9-10.9)
Total Cholesterol (mmol/l)	6.0 (5.0-7.1)	5.9 (5.7-6.5)	5.6 (4.6-6.7)	6.0 (4.6-6.7)
HDL cholesterol (mmol/l)	1.86 (1.70-2.30)	2.10 (1.48-2.24)	1.96 (1.76-2.12)	1.88 (1.68-2.02)
LDL Cholesterol (mmol/l)	3.7 (2.9-4.6)	3.4 (2.6-3.9)	3.2 (2.5-4.0)	3.6 (2.9-4.4)

All values are median (IQR)

Table 11: Baseline physical function and cardiovascular parameters by treatment groups.

	50 000IU	150 000IU	500 000IU	Control
	(n=11)	(n=11)	(n=11)	(n=27)
Repeated chair stand score	3 (3-4)	2 (2-4)	4 (2-4)	3 (2-4)
Narrow walk score	4 (4-4)	4 (3-4)	4 (4-4)	4 (4-4)
Balance test score	4 (4-4)	4 (4-4)	4 (4-4)	4 (4-4)
Overall SPPB score	11 (11-12)	10 (9-12)	12 (10-12)	11 (10-12)
Grip Strength (kg)	22.7 (17.6-24.0)	20.8 (17.5-24.4)	20.9 (17.5-24.1)	20.2 (17.4-23.6)
Laying systolic BP (mmhg)	130 (126-139)	139 (130-163)	138 (126-159)	131 (120-144)
Laying diastolic BP (mmhg)	69 (67-75)	78 (73-82)	73 (67-79)	73 (66-79)
Standing systolic BP (mmhg)	133 (125-136)	129 (117-153)	141 (125-154)	136 (120-149)
Standing diastolic BP (mmhg)	78 (73-79)	80 (74-86)	78 (71-84)	75 (64-84)
Laying pulse (bpm)	67 (63-81)	80 (69-84)	66 (57-67)	65 (57-76)
Standing pulse (bpm)	88 (74-97)	88 (83-90)	74 (69-84)	75 (68-83)

All values are median (IQR)

Table 12: Baseline demographics, dietary intake and sunlight exposure score for treatment groups combined vs. control group and p-values for between-group differences.

	Combined Treatment	Control	P-value
Age (Years)	62 (58-68)	60 (58-64)	.461
Height (cm)*	160.5 (+/-5.2)	160.6 (+/-7.1)	.921
Weight (kg)*	64.5 (+/-7.9)	66.7 (+/-8.4)	.312
BMI (kg/m ²)	23.8 (22.8-27.9)	26.4 (23.0-28.7)	.393
Total dietary vitamin D intake (µg/day)	1.5 (1.1-2.8)	2.2 (0.9-3.2)	.822
Total dietary calcium intake (mg/day)*	817 (+/-262)	813 (+/-238)	.966
Sunlight exposure score	83 (42-104)	80 (54-110)	.674

Values are median and IQR unless stated

*Values presented as mean and SD where Shapiro-Wilk test for normality $P > 0.05$ in both groups and independent samples t-test used to determine between group differences (otherwise a Mann-Whitney U test)

3.1 Between-group comparisons for baseline demographics

There was no significant difference in participant age, height, weight and BMI between the treatment groups combined and the control group (**table 12**). The mean age of the groups indicated that participants on the study were a relatively 'young' older age cohort. The median BMI of the treatment group and control group indicates that participants were in the 'normal weight' and 'pre-obese' range, respectively.

3.2 Between-group comparisons for dietary vitamin D intake and sunlight exposure scores

There was no significant difference in total dietary vitamin D intake or dietary calcium intake between the treatment groups combined and the control group (**table 12**). Dietary vitamin D intake was only 1.5% and 2.2% of the reference nutrient intake (RNI) of 10ug/day in the UK (3). This is lower than the mean intake of vitamin D from dietary sources only of 3.4% (112IU) of the RNI reported for women aged 19-64 years in the latest UK National Diet and Nutrition Survey (3).

Dietary intake of calcium was above the RNI of 700mg/day for those aged 50 years and over in both the treatment groups combined and the control group (**table 12**) and was comparable to the mean reported intake for dietary calcium of 710mg/day from the NDNS for 19-64 year olds in the UK (PHE, 2018).

There was no significant difference in sunlight exposure score between the treatment groups combined and the control group (**table 12**). Sunlight exposure scores were comparable to scores derived in another study carried out by our group in Sheffield that used the same sunlight exposure questionnaire (20).

3.3 Baseline total 25(OH)D

Total 25(OH)D by immunoassay was lower in the treatment groups combined compared to the control group, as dictated by the study design (**table 13**). By immunoassay, baseline total 25(OH)D was slightly higher than screening total 25(OH)D. Total 25(OH)D at baseline was measured by CLIA (ImmunodiagnosticSystems, Boldon, UK) at the Bone Biochemistry Laboratory (University of Sheffield, UK). Total 25(OH)D at screening was measured using a Cobas e411 autoanalyser (Roche Diagnostics, Mannheim, Germany) at Laboratory Medicine (NGH, UK). Therefore, discrepancies between total 25(OH)D values at screening and baseline are likely due to the differences in measurement methods. Measurement of total 25(OH)D₃ by LC-MS/MS confirmed that total 25(OH)D₃ was below 30nmol/l in treated participants (**table 13**). Total 25(OH)D₂ was undetectable (<10nmol/l) in all but one participant.

3.4 Between-group baseline comparisons of free 25(OH)D

Measured free 25(OH)D, calculated free 25(OH)D (derived using total 25(OH)D measured by immunoassay) and calculated free 25(OH)D₃ (derived using total 25(OH)D measured by LC-MS/MS) were all significantly lower in the treatment groups combined when compared to the control group. This is expected as free 25(OH)D is strongly correlated with total 25(OH)D in healthy individuals in normal physiologic conditions and across a wide range of total 25(OH)D concentrations (165).

Measured free 25(OH)D in the treatment groups combined and the control group were similar to the calculated concentrations. Calculated free 25(OH)D was slightly higher than the directly measured free 25(OH)D concentration, but this has also been reported in other studies (304).

The control group measured free 25(OH)D (median: 13.8pmol/l [IQR: 9.9-15.2]), calculated free 25(OH)D (median: 15.7pmol/l [IQR: 12.3-20.6]) and calculated free 25(OH)D₃ (median: 14.3pmol/l [IQR: 10.5-17.0]) were similar to other reports in healthy vitamin D sufficient participants (20, 165, 305).

Percentage free 25(OH)D was consistent across all ratios of free 25(OH)D to total 25(OH)D in the treatment groups combined and in the control group, although the percentage free derived from the calculated free 25(OH)D₃ to total 25(OH)D₃ was significantly lower in the treatment groups combined vs. the control group.

Variation in calculated free 25(OH)D is influenced by the methods of measurement for total 25(OH)D (e.g. LC-MS/MS vs. immunoassay) and VDBP (polyclonal vs. monoclonal antibody assays) that have been used in the calculation. The calculated free 25(OH)D percentage has been reported to be between 0.02% and 0.09% of total 25(OH)D concentrations, with values ranging from 1.3pmol/l to 20.3pmol/l in 95% of healthy individuals (156, 305), or around 0.044% in healthy Caucasian individuals when a polyclonal assay for VDBP was used (165).

3.5 Between-group baseline comparisons of 1, 25(OH)₂D and free 1, 25(OH)₂D

Total 1, 25(OH)₂D and free 1, 25(OH)₂D were significantly lower in the treatment groups combined compared to the control group. The median percent free (calculated free 1, 25(OH)₂D: total 1, 25(OH)₂D) in the treatment groups combined and the control groups are similar to previously reported percent free 1, 25(OH)₂D in healthy participants of 0.42% (±0.07) (306).

Table 13: Baseline biochemical parameters for treatment groups combined vs. control group and P-values for between-group differences.

	Combined Treatment	Control	P-value
Total 25(OH)D (nmol/l)*	32.4 (+/-8.3)	70.4 (+/-20.1)	
25(OH)D ₃ (nmol/l)	20.1 (17.2-24.5)	54.8 (44.1-67.9)	<.001
Measured free 25(OH)D (pmol/l)	6.6 (5.5-7.2)	13.8 (9.9-15.2)	<.001
Calculated free 25(OH)D (pmol/l)	7.8 (6.4-9.4)	15.7 (12.3-20.6)	<.001
Calculated free 25(OH)D ₃ (pmol/l)	4.6 (4.1-6.3)	14.3 (10.5-17.0)	<.001
Percent free m25(OH)D (measured f25(OH)D: total 25(OH)D)	0.020 (0.016-0.027)	0.019 (0.015-0.027)	.364
Percent free c25(OH)D (calculated f25(OH)D: total 25(OH)D)	0.024 (0.019-0.032)	0.022 (0.017-0.026)	.592
Percent free c25(OH)D ₃ (Calculated f25(OH)D ₃ : total 25(OH)D ₃)	0.014 (0.018- 0.026)	0.026 (0.021-0.032)	.018
Total 1, 25(OH) ₂ D (pmol/l)	65 (49.8-81.5)	81.0 (63.0-105.5)	.005
Calculated free 1, 25(OH) ₂ D (fmol/l)*	283 (+/-100)	380 (+/-137)	.006
Percent free 1, 25(OH) ₂ D	0.44 (0.35-0.57)	0.46 (0.36-0.60)	.800

Values are median and IQR unless stated. Significant P-values (P<0.05) in bold.

*Values presented as mean and SD; Shapiro-Wilk test for normality P>0.05 and independent samples t-test used to determine between group differences (otherwise a Mann-Whitney U test)

Table 13 (cont): Baseline biochemical parameters for treatment groups combined vs. control group and P-values for between-group differences.

	Combined Treatment	Control	P-value
PTH (pg/ml)*	41.0 (+/-12.3)	33.3 (+/-10.9)	.016
Serum calcium (mmol/l)*	2.33 (+/-0.07)	2.33 (+/-0.08)	.861
Ionized calcium (mmol/l)	1.24 (1.22-1.27)	1.24 (1.23-1.28)	.538
Serum Phosphate (mmol/l)*	1.20 (+/-0.12)	1.17 (+/-0.11)	.364
Serum Creatinine (μmol/l)*	65 (+/-8)	67 (+/-12)	.531
24hr urine Ca (mmol/24hrs)	2.6 (1.6-3.4)	2.8 (2.0-4.8)	.406
24hr urine Cr (mmol/24hrs)*	7.8 (+/-1.8)	8.7 (+/-1.8)	.078
FGF-23 (pg/ml)	44.9 (38.9-52.9)	43.1 (33.6-47.3)	.319
Albumin (g/L)	47 (46-49)	47 (45-49)	.946
VDBP (μg/ml)*	307 (+/-53)	305 (+/-54)	.864
PINP (ng/ml)	52.8 (45.0-66.3)	57.2 (47.4-66.7)	.941
Osteocalcin (ng/ml)	24.4 (21.1-30.9)	25.0 (21.4-32.3)	.806
CTX (ng/ml)*	0.51 (+/-0.19)	0.47 (+/-0.15)	.333

*Values presented as mean and SD; Shapiro-Wilk test for normality $P > 0.05$ and independent samples t-test used to determine between group differences (otherwise a Mann-Whitney U test).

Table 13 (cont): Baseline biochemical parameters for treatment groups combined vs. control group and P-values for between-group differences.

	Combined Treatment	Control	P-value
Renin (ng/ml/hr)	2.5 (1.4-3.3)	2.5 (1.7-4.2)	.592
Aldosterone (ng/dl)*	8.2 (+/-4.7)	7.9 (+/-2.7)	.745
Total Cholesterol (mmol/l)*	5.8 (+/-1.0)	6.0 (+/-1.0)	.454
HDL cholesterol (mmol/l)*	1.93 (+/-0.41)	1.87 (+/-0.34)	.480
LDL Cholesterol (mmol/l)*	3.4 (+/-0.9)	3.7 (+/-1.0)	.248

Values are median and IQR unless stated. Significant P-values ($P < 0.05$) in bold.

*Values presented as mean and SD; Shapiro-Wilk test for normality $P > 0.05$ and independent samples t-test used to determine between group differences (otherwise a Mann-Whitney U test)

3.6 Between-group baseline comparisons for other calcium and vitamin D related biochemistry

As expected, PTH was significantly higher in the 25(OH)D deficient combined treatment group at baseline compared to the 25(OH)D sufficient control group. PTH is typically lower in vitamin D sufficiency compared to those who are vitamin D deficient. Nonetheless, PTH was in the reference range of 10 - 65ng/ml for both the treatment groups combined and the control group.

Serum calcium, ionised calcium, phosphate and creatinine were not significantly different between the combined treatment groups and the control group and were within the healthy reference range.

Twenty-four hour urinary calcium and creatinine excretion were not significantly different between the combined treatment group and the control group, and were within the healthy reference range.

Serum albumin and VDBP were not significantly different between the two groups. Serum albumin was within the reference range (35-50g/L). FGF-23 was also not significantly different between the combined treatment group and control group.

A summary of baseline values and between-group comparisons for calcium and vitamin D associated biochemistry can be found in **table 13**.

3.7 Between-group comparisons for baseline bone turnover markers

Serum PINP, CTX, and OC were not significantly different between the combined treatment groups and the control group. The reference range for PINP in vitamin D sufficient postmenopausal women aged 60-75 years is 8.2 – 102.3ng/ml and for CTX the reference interval is 0.09 - 1.05ng/ml (307). The median value of PINP in 60-64 year old females is 47.4ng/ml (IQR: 35.2-59.8) and the median value for CTX is 0.32ng/ml (IQR: 0.23-0.51), calculated from a sub-set of 114 post-menopausal women (307). The median serum OC concentration in postmenopausal women is 18.6ng/mL (IQR: 13.6–25.6). In the 60-64 years age group the median value for OC is 17.9ng/ml (IQR: 13.5 - 22.8) (308). The reference interval for osteocalcin in postmenopausal women is 8.0 - 40.9ng/ml (308).

All bone turnover markers in this study fell within the relevant reference range of each marker for healthy post-menopausal women.

A summary of baseline values and between-group comparisons for bone turnover markers is shown in **table 13**.

3.8 Between-group comparisons for cardiovascular measurements

Total cholesterol, HDL cholesterol and LDL cholesterol did not differ between the treatment group and control group. Total cholesterol was slightly higher in both groups than the recommended upper value of 5.2mmol/l. HDL cholesterol was slightly above the lower recommended limit for women of 1.3mmol/l in both groups. LDL

cholesterol was slightly higher in the control group than the recommended upper limit of 3.4mmol/l in healthy individuals. Renin and aldosterone did not differ between groups.

Median supine systolic blood pressure was in the high-normal (120-140mmHg) range in both the combined treatment group and control group. Supine diastolic blood pressure was in the normal range (60-85mmHg) in both groups. There were no significant between-group differences for any measure of blood pressure.

Supine pulse rate was significantly higher in the control group compared to the treatment group, although the difference between groups was small. Resting pulse rate was within the normal range for healthy adults (60-100bpm). Standing pulse rate was not significantly different between groups.

A summary of baseline values and between-group comparisons for cardiovascular-associated biochemistry can be found in **table 13**. A summary of baseline values and between-group comparisons for blood pressure and pulse rate can be found in **table 14**.

3.9 Between-group comparisons for physical function measures

There were no significant between-group differences in overall SPPB scores or scores for the individual components of the SPPB. Scores on these tests were high when compared to scores generated by frail or elderly population in other studies, reinforcing that participants on the study were overall relatively healthy (10, 212).

There was also no significant between-group difference in grip strength score. Grip strength scores were slightly lower than reported normative values of approximately 25-26 kg for postmenopausal women (309, 310).

A summary of baseline values and between-group comparisons for physical function assessments is shown in **table 14**.

Table 14: Baseline physical function and cardiovascular parameters for treatment groups combined vs. control group and P-values for between-group differences.

	Combined Treatment	Control	P-value
Repeated chair stand score	3 (2-4)	3 (2-4)	.702
Narrow walk score	4 (4-4)	4 (4-4)	.429
Balance test score	4 (4-4)	4 (4-4)	.679
Overall SPPB score	11 (10-12)	11 (10-12)	.703
Grip Strength (kg)*	21.5 (+/-4.9)	20.4 (+/-4.5)	.385
Laying systolic (mmhg)	135 (128-147)	131 (120-144)	.444
Laying diastolic (mmhg)	73 (68-79)	73 (66-79)	.547
Standing systolic (mmhg)	133 (124-145)	136 (120-149)	.888
Standing diastolic (mmhg)	78 (73-84)	75 (64-84)	.812
Laying pulse (bpm)*	72 (+/-12)	66 (+/-11)	.049
Standing pulse (bpm)*	83 (+/-12)	77 (+/-15)	.100

Values are median and IQR unless stated. Significant P-values ($P < 0.05$) in bold.

*Values presented as mean and SD; Shapiro-Wilk test for normality $P > 0.05$ and independent samples t-test used to determine between group differences (otherwise a Mann-Whitney U test)

Chapter 4:

Change in vitamin D metabolites after
bolus dose supplementation

Chapter overview

This chapter will address changes in total and free 25(OH)D and total and free 1, 25(OH)D (research questions 1 and 2) after administration of the different oral bolus doses of vitamin D₃ in vitamin D deficient post-menopausal women. We hypothesised that the influx of vitamin D into circulation may overwhelm the carrier proteins for vitamin D in circulation, leading to a disproportionate rise in levels of free vitamin D metabolites (percent free) which could cause adverse effects. A greater understanding of changes to these metabolites after bolus dosing will further our understanding of the potential role they play in vitamin D toxicity.

4.1 Research Questions

1. How do different bolus doses of vitamin D affect total 25(OH)D, free 25(OH)D and 1, 25(OH)₂D in vitamin D deficient postmenopausal women?
2. Is there a disproportionate rise in free 25(OH)D and or free 1, 25(OH)₂D compared to total 25(OH)D and 1, 25(OH)₂D after bolus dose vitamin D supplementation?

4.2 Aims

1. To determine the response of the total 25(OH)D and 1, 25(OH)₂D to different bolus doses of vitamin D₃
2. To determine the response of free 25(OH)D and free 1, 25(OH)₂D to different bolus doses of vitamin D₃
3. To investigate whether free 25(OH)D rises in a disproportionate manner after bolus dosing
4. To investigate whether free 1, 25(OH)₂D rises in a disproportionate manner after bolus dosing

4.3 Hypotheses

1. There will be a dose-response rise in total 25(OH)D and 1,25(OH)₂D after bolus dosing of vitaminD₃ by week 1
2. There will be a dose-response rise in free 25(OH)D and free 1,25(OH)₂D after bolus dose supplementation of vitamin D₃ by week 1
3. There will be a disproportionate rise in free 25(OH)D in response to the largest dose of vitamin D₃ (500 000IU) 1 week after administration
4. There will be a disproportionate rise in free 1, 25(OH)₂D in response to the larger bolus dose of vitamin D₃ (500 000IU) 1 week after administration

4.4 Effect of oral bolus dosing on total 25(OH)D concentration

A limited number of studies have investigated the effect of a single, oral bolus dose of vitamin D₃ (or with a dosing frequency of \geq 3-months) on biochemical parameters of calcium and vitamin D metabolism in healthy people. A summary of these published studies is found in **table 15**.

4.5 Effect of a single, large oral bolus dose of vitamin D₃ on total 25(OH)D concentration

It is clear that a single large bolus dose of vitamin D₃ is effective at rapidly increasing serum total 25(OH)D concentrations across many population sub-groups (8, 172, 173, 200, 201, 214-216, 218, 219, 237, 245, 286, 311-313). After a bolus dose, total 25(OH)D increases in a dose-dependent manner in healthy population groups (219). The absolute increase in total 25(OH)D from baseline varies across studies and depends on a variety of factors, including baseline 25(OH)D status, the dose administered, population under study, measurement method of total 25(OH)D and the time points captured within the study. The heterogeneity in study design makes these studies difficult to compare. An overview of the increase in total 25(OH)D after a range of bolus doses of vitamin D₃ is given below and in **table 15**.

4.5.1 Bolus doses < 200 000IU

Single bolus doses of vitamin D₃ of 100 000IU appear to increase total 25(OH)D concentration 1-week after administration by approximately 13-46nmol/l from baseline in healthy vitamin D sufficient individuals (219, 245, 286, 311). Total 25(OH)D levels appear to remain above the sufficiency threshold at 3-4 months after administration of 100 000-150 000IU of vitamin D₃ (200, 219, 237, 286, 311).

4.5.2 Bolus doses \geq 200 000IU and < 300 000IU

The most commonly used bolus dose of vitamin D₃ across studies is 300 000IU. After a 200 000-300 000IU bolus of vitamin D₃, total 25(OH)D increases from baseline values by approximately 11-31nmol/l up to 1-week after administration in healthy vitamin D sufficient participants (219, 311). Larger increases in total 25(OH)D from baseline have been demonstrated in vitamin D deficient and insufficient participants after a 250 000-300 000IU vitamin D₃ bolus, ranging from approximately +62nmol/l at 5-days (218) to between +16-120nmol/l after 4-6 weeks (172, 201, 215, 312) and approximately +28-75nmol/l 3-months post-administration (201, 216, 217).

After a 200 000-300 000IU vitamin D₃ bolus, total 25(OH)D concentration remains above the sufficiency threshold up to 3-months after administration in baseline vitamin D deficient, insufficient and sufficient participants (201, 216, 217, 311). Total 25(OH)D levels in some studies are still elevated from baseline levels up to 6-months after

administration of 300 000IU of vitamin D₃ (215, 216), although total 25(OH)D may fall below the insufficiency threshold in some population sub-groups (201, 215).

4.5.3 Bolus doses > 500 000IU

Larger dose bolus dosing strategies have also been administered. After a 500 000-600 000IU oral vitamin D₃ bolus, there is a rapid increase in total 25(OH)D concentration of between +143-153nmol/l by 3-days post-administration and approximately +116-118nmol/l at 1-month after administration in healthy vitamin D insufficient younger adults (214, 313). Increases in total 25(OH)D from baseline in older vitamin D sufficient participants are smaller. Total 25(OH)D concentration rises from baseline values by +113nmol/l at 3-days post-administration (219) and between approximately 31-75nmol/l at 1-month after administration (8, 173, 213, 219) in vitamin D sufficient older adults. Total 25(OH)D concentrations appear to remain elevated from baseline by approximately 25-70nmol/l at 3-months post-administration in baseline vitamin D insufficient and sufficient older adults (8, 213, 219, 312). In vitamin D sufficient older adults total 25(OH)D is reported to still be above baseline levels at 9-months after administration (213), but another study in a similar population sub-group has showed a return to baseline levels at just 4-months post administration (173).

Table 15: Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose ≥ 600 000IU												
Cipriani et al., (2010) (214)	48	Healthy young adults aged: 36 +/- 8	M/F	600 000IU D ₃	Single oral bolus dose	40 +/- 16	193 +/- 76.3	3-, 15- & 30 days 3-days	156 +/- 65	30 days	RIA DiaSorin	Serum 25(OH)D, PTH, 1, 25(OH) ₂ D & sCa
Cipriani et al., (2013) (173)	24 (n = 6 per group)	Healthy participants aged: 63.9 +/- 7.1	M/F	(1) 600 000IU D ₂ (2) 600 000IU D ₃ (3) 600 000IU D ₂ (4) 600 000IU D ₃	(1) Single oral dose (2) Single oral dose (3) Single IM dose (4) Single IM dose	(1) 52.2 +/- 14.8 (2) 52 +/- 16.3 (3) 37.8 +/- 11.0 (4) 69.5 +/- 14.3	(1) +15 +/- 11 basal increase (2) +31 +/- 11.25 basal increase (3) +28 +/- 7 basal increase (4) +28 +/- 16 basal increase	30-, 60-, 90- & 120-days (1) 30-days (2) 30-days (3) 120-days (4) 120-days	(1) Approx. baseline levels (2) Approx. baseline levels (3) +28 +/- 7 basal increase (4) +28 +/- 16 basal increase	120 days	RIA Diasorin	Total 25(OH)D, 1,25(OH) ₂ D, 24, 25(OH) ₂ D
Cipriani et al., (2013) (313)	18	32.1 +/- 4.7 previously undergone surgery for localised melanoma	F	600 000IU D ₃	Single oral bolus dose	44.5 +/- 18.5	187.8 +/- 41.5	3-, 15-, 30-, 60- & 90-days 3 days	79.3 +/- 33.3	90 days	RIA Diasorin	Total 25(OH)D, 1, 25(OH) ₂ D, PTH, siCa, sPh

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; * values shown are median and range, *Values shown are median and IQR

Table 15 (cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample Size	Age (years) & Health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose		25(OH)D Assay
Single large bolus dose ≥ 600 000IU												
Tellioglu et al., (2012) (212)	66	(1) 75.3 +/- 7.5 (2) 75.5 +/- 6.1 Ambulatory nursing home participants	M/F	(1) 600 000IU D ₃ (n = 32) (2) 600 000IU D ₃ (n = 34)	(1) Single oral dose (2) Single IM dose	(1) 37.2 +/- 13.8 (2) 29.4 +/- 19.0	(1) 118.9 +/- 31.8 (2) 130.9 +/- 35.5	6 weeks & 12 weeks (1) 6 weeks (2) 12 weeks	(1) 107.4 +/- 33.5 (2) 130.9 +/- 35.5	12 weeks	Unknown	Total 25(OH)D, sCa, PTH & SPPB
Rossini et al., (2012) (219)	61	Elderly community dwelling aged: 75 +/- 3	M/F	(1) 600 000IU D ₃ (n= 12) (2) 300 000IU D ₃ (n= 12) (3) 100 000IU D ₃ (n= 13) (4) Control (n= 24)	Single oral bolus doses	(1) 54 +/- 14 (2) 65 +/- 17 (3) 66 +/- 19 (4) 55 +/- 18	(1) 167 +/- 43 (2) 96 +/- 23 (3) 85 +/- 21 (4) -	1-, 3-, 7-, 14-, 30-, 60- & 90- days (1) 3-days (2) 7-days (3) 7-days (4)-	(1) 88 +/- 14 (2) 72 +/- 15 (3) 79 +/- 20 (4) 58 +/- 15	90 days	CLIA	Total 25(OH)D PTH CTX

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, *Values shown are median and IQR

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen			Serum 25(OH)D profile				Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose ≥ 500 000IU												
Sanders et al., (2010) (8)	2256 (n = 131 subset for 25(OH)D analysis)	Community dwelling aged: 76 (77-83) ⁺	F	(1) 500 000IU D ₃ (n=74) vs. (2) placebo (n = 58)	Annual oral bolus dose for 5 years	(1) 53 (40-65) ⁺ (2) 45 (40-57) ⁺	(1) Approx. 125 (2) Approx. 30	1-, 3- & 12-months (1) 1 month	(1) Approx. 80 (2) Approx. 45	12-months	RIA DiaSorin	Total 25(OH)D
Bacon et al., (2009) (213)	63	82+/- 7 recently hospitalized	M / F	(1) 500 000IU D ₃ (n = 19) vs. (2) 500 000IU D ₃ + 50 000IU (n = 22) per month vs. (3) 50 000IU D ₃ (n = 22)	(1) Single oral bolus (2) single oral bolus + monthly (3) monthly	(1) 58 +/- 25 (2) 66 +/- 41 (3) 50 +/- 25	(1) Approx. 120 (2) Approx. 120 (3) Approx. 80	1-, 2-, 3-, 4-, 5-, 6-, 7-, 8 & 9-months (1) 1-month (2) 1-month (3) 5-months	(1) 69 +/- 5 (2) 91 +/- 4 (3) 80 +/- 5	9 months (1-month in the 50 000IU monthly group (3))	RIA DiaSorin	25(OH)D, PTH and PINP & grip strength

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; * values shown are median and range, ⁺Values shown are median and IQR

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose ≥200 000IU ≥ 300 000IU												
Leventis & Kiely (2009) (215)	69	Vitamin D insufficient aged: (1) 53 (29-82)* (2) 43 (23-72)*	M/F	(1) 300 000IU D ₂ (n = 50) (2) 300 000IU D ₃ (n = 19)	(1) single IM dose (2) Single oral dose	(1) 20.3 (17-35)* (2) 27 (5-40)*	(1) 35.2 (17-50)* (2) 134.6 (65-214)*	6-, 12- & 24-weeks (1) 12-weeks (2) 6-weeks	(1) 35.2 (17-50)* (2) 42.8 (23-78)*	24-weeks	IDS: ELISA	Total 25(OH)D, PTH, sCa, sPh
Giusti et al., (2010) (217)	60	Community dwelling older adults aged: (1) 74 +/- 6 (2) 71 +/- 4	F	(1) 300 000IU D ₃ (2) 1000IU D ₃	(1) 3-monthly oral bolus dose (2) daily oral dose	(1) 23 +/- 12 (2) 23 +/- 13	(1) 52 +/- 32 (2) 58 +/- 19	3- & 6-months (1) 3-months (2) 6-months	(1) 52 +/- 32 (2) 58 +/- 19	6-months	RIA Diasorin	Total 25(OH)D, PTH, sCa, UrCa (24hr), CTX,
Cavalcante et al., (2015) (312)	40	Elderly aged: 68 +/- 6 years		(1) 200 000IU D ₃ (n = 20) vs. (2) Placebo (n = 20)	(1) Single oral dose (2) Single oral dose	(1) 63 +/- 7 (2) 67 +/- 9	(1) 79 +/- 15 (2) 61 +/- 10	4-weeks only	(1) 79 +/- 15 (2) 61 +/- 10	4-weeks	HPLC	Total 25(OH)D, PTH, sCa, blood pressure
Von Restorff et al., (2009) (216)	33	Elderly with 25(OH)D <25nmol/l aged: 81 +/- 6	M/F	300 000IU D ₃	Single oral bolus dose	15 +/- 6	81 +/- 30	3- and 6-months 3-months	69 +/- 18	6-months	RIA Diasorin	Total 25(OH)D, PTH & sCa

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, +Values shown are median and IQ

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose ≥200 000IU ≥ 300 000IU (cont)												
Romagnoli et al., (2008) (172)	32 (n = 8 per group)	Elderly nursing home residents aged: 66-97 (1) 79 +/- 8 (2) 80 +/- 10 (3) 81 +/- 5 (4) 79 +/- 5	F	(1) 300 000IU D ₃ (2) 300 000IU D ₂ (3) 300 000IU D D ₃ (4) 300 000IU D ₂	(1) Single oral bolus (2) Single oral bolus (3) Single IM (4) Single IM	(1) 33 +/- 25 (2) 32 +/- 23 (3) 21 +/- 9 (4) 18 +/- 7	(1) +120 basal difference (2) + ~80 basal difference (3) +65 basal difference (4) +23 basal difference	3-, 7-, 30- & 60-days (1) 30-days (2) 3-days (3) 60-days (4) 60-days	(1) +70 basal difference (2) + 25 basal difference (3) +65 basal difference (4) +23 basal difference	60 days	RIA DiaSorin	25(OH)D, 1, 25(OH) ₂ D & PTH
Premaor et al., (2008) (201)	28	Low socioeconomic elderly with secondary hyperparathyroidism aged: 81 +/- 9	M / F	(1) 300 000IU D ₃ (n = 14) vs. (2) 800IU D ₃ (n = 14)	(1) single oral bolus (2) daily	(1) 31 +/- 17 (2) 23 +/- 12	(1) Approx. 80 (2) Approx. 48	1-, 2-, 3-, 6-, & 9-months (1) 1-month (2) 2-months	(1) Approx. 30 (2) Approx. 35	9-months	LIAISON Chemiluminescence DiaSorin	Total 25(OH)D, PTH & sCa
Valimaki et al., (2016) (311)	60	75 +/- 2.9	F	(1) 100 000IU D ₃ (n = 20) or; (2) 200 000IU D ₃ (n = 20) (3) Placebo (n = 20)	Every three months for 1-year	(1) 60.0 (31-99)* (2) 63.5 (38-87)* (3) 61.5 (15-101)*	(1) 73.0 (59-119)* (2) 74.5 (58-114)* (3) 60.0 (18-102)*	1 week & 3 months (1) 1 week (2) 1 week	(1) 67.0 (51-106)* (2) 71.0 (49-92)* (3) 64.0 (34-92)*	3-months (total study period was 1 year)	CLIA IDS-iSYS	Total 25(OH)D, 1, 25(OH) ₂ D, PTH, siCa, urCa, bone turnover markers

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, +Values shown are median and IQ

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose ≥200 000IU ≥ 300 000IU (cont)												
Kearns et al., (2015) (218)	28	Young, healthy adults aged: 66-97 (1) 28.2 +/- 6.7 (2) 26.5 +/- 5.2	M/F	(1) 250 000IU D ₃ (n = 14) (2) Placebo (n = 14)	(1) Single oral bolus (2) Single oral dose	(1) 41 (2) 41	(1) 103 (95% CI: 87-122) (2) 45 (95% CI: 37-53)	5-, 90- & 365-days (1) 5-days (2) 5-days	Both treatment and placebo not significantly different to baseline at 90 and 365-days	365-days	CLIA IDS-iSYS	25(OH)D, & PTH

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, *Values shown are median and IQ

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose < 200 000IU												
Glendenning et al., (2012) (237)	686 (total 25(OH)D measured in a sub-set of 40)	Community dwelling ambulant women aged: (1) 76.9 +/- 4.0 (2) 76.5 +/- 4.0	F	(1) 150 000IU D ₃ (n = 20) or; (2) Placebo (n = 20)	Every three months for 1-year	(1) 65.0 +/- 17.8 (2) 66.5 +/- 27.1	(1) 74.0 +/- 21 (2) 59.8 +/- 20.5	3-months (6-months and 9-months time points were after additional doses administered at 3- and 6-months)	(1) 74.0 +/- 21 (2) 59.8 +/- 20.5	3-months (total study period was 9-months)	RIA Diasorin	Total 25(OH)D, grip strength and falls
Ilahi et al., (2008) (286)	40	Older aged: 70.8 +/- 5.6 & Younger aged: 37.9 +/- 8.0 Control aged: 71.1 +/- 9.8	M/F	(1) 100 000IU D ₃ (n = 30) (2) control (n = 10)	(1) Single oral bolus dose (2) -	(1) 68 +/- 19 (2) 68 +/- 19	(1) 105 +/- 23 (2) 105 +/- 23	1-, 2-, 3-, 4-, 5-, 6-, 7-, 14-, 21-, 28-, 42-, 56-, 70-, 84-96- & 112- days (1) 7-days	(1) 74 +/- 10 (2) 74 +/- 10	112-days	RIA IDS	Total 25(OH)D & hypercalcaemia
Rosseau et al., (2015) (245)	49	(1) Healthy participants vs. (2) adults burns patients aged: 26 (22–60)*	M/F	100 000IU D ₃ (1) n = 29 (2) n = 20	Single bolus dose	(1) 53.8 (25.3-109.0)* (2) 27.5 (4.5-78.5)*	(1) 99.8 (64.5-167)* (2) 48.3 (12.5-83.5)*	1-week	(1) 99.8 (64.5-167)* (2) 48.3 (12.5-83.5)*	1-week	LC-MS/MS	Total 25(OH)D, Calculated free 25(OH)D, free fraction 25(OH)D, 1, 25(OH) ₂ D, PTH, sCa, siCa & FGF-23

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, *Values shown are median and IQ

Table 15 (Cont): Summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured total 25(OH)D and/or 1, 25(OH)₂D.

Reference	Participant Characteristics			Dosing regimen		Serum 25(OH)D profile					Outcomes	
	Sample size	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Peak total 25(OH)D	Follow-up time points & peak total 25(OH)D	Total 25(OH)D at end of study or before a second dose (nmol/l)	End of study time point or before a second dose	25(OH)D Assay	Outcome measures of interest
Single large bolus dose < 200 000IU												
(cont)												
Pekkarinen et al.,(2010)	40	Community dwelling older participants from Finland aged; (1) 74.1 (71.2-78.0) * (2) 73.6 (69.3-78.8) *	F	(1) 97333IU D ₃ vs. (2) 800IU	(1) Bolus dose every 4-months vs. (2) 400IU twice daily	(1) 57.5 (17.0-84.0) * (2) 54.0 (40.0-81.0) *	(1) Approx. 68nmol/l (2) Approx. 80nmol/l	1-, 2-, 3-, 4-, 6-, 8-, 10- & 12-months (1) 1-month (2) 3-months	(1) Approx. 55nmol/l (2) Approx. 70nmol/l	4-months (total study period was 12-months)	HPLC	Total 25(OH), PTH, PINP, sCa & uCa

Time point of peak total 25(OH)D are shown in bold. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range, *Values shown are median and IQR.

4.5.4 Efficacy of oral bolus doses of vitamin D₃ vs. vitamin D₂ on total 25(OH)D

It appears that bolus doses of vitamin D₃ are more effective at increasing total 25(OH)D levels than bolus dose vitamin D₂ (172, 173). After bolus dosing of either 300 000IU of vitamin D₂ or D₃ in vitamin D insufficient elderly nursing home residents (n=8 per group), there was a similar basal increase in total 25(OH)D in both groups 3-days after administration, demonstrating comparative absorption and 25-hydroxylation (172). However, at 7-days, 30-days and 60-days after administration there was a decrease in total 25(OH)D concentration in the vitamin D₂ treatment group, with levels returning to just above 50nmol/l (~+25nmol/l basal difference) before the end of study period. In contrast, there were steady increases in total 25(OH)D concentration in the vitamin D₃ treatment group up to 30-days post-administration and total 25(OH)D still remained well above the insufficiency threshold at 60-days (~+70nmol/l basal difference) (172).

In a similar study in vitamin D sufficient healthy older participants (n = 6 per group), vitamin D₃ was also more effective at increasing total 25(OH)D after a 600 000IU bolus than vitamin D₂. The mean basal increase in total 25(OH)D in the D₃ treatment group was approximately double than reported in the D₂ treatment group 30-days after administration (approximately + 31nmol/l and +15nmol/l, respectively) (173). Total 25(OH)D in the D₃ treated group remained significantly above baseline 90-days after administration, but had returned to baseline by 60-days post-administration in the D₂ treated group (173).

The greater efficacy of vitamin D₃ over vitamin D₂ at increasing and maintaining total 25(OH)D when administered as a bolus dose may reflect a more rapid metabolism and clearance of the D₂ metabolite. Indeed, it has been demonstrated that 25(OH)D₂ half-life is shorter than that of 25(OH)D₃ (130). There are several factors that may contribute to this theory, such as higher affinities of the D₃ metabolites for VDBP and/or the vitamin D receptor or a lower affinity of D₃ metabolites to the 24-hydroxylase for the catabolic pathways.

4.6 Effect of a single, large oral bolus dose of vitamin D₃ on total 1, 25(OH)₂D

Only five bolus dosing studies to date have reported 1, 25(OH)₂D measurements after bolus dose D₃.

4.6.1 Bolus doses $\geq 100\ 000\text{IU}$ and $\leq 300\ 000\text{IU}$

After a 100 000IU bolus of vitamin D₃ in healthy participants there was no significant change in 1, 25(OH)₂D 1-week after administration (baseline: 82.6pg/ml, [range: 40.2-150.3] vs 1-week: 79.7pg/ml [range: 41.6-137.7])(245). These median values are within the normal range for this metabolite (23–109 pg/ml).

Valimaki et al reported changes in 1, 25(OH)₂D in community dwelling older women after a 100 000IU or 200 000IU D₃ bolus every three months for a year compared to placebo (311). There were no significant within-group changes from pre- to post supplementation in 1, 25(OH)₂D at 1-week after administration. There were also no

between-group differences in 1, 25(OH)₂D over time for the whole of the study period (311). Questions are raised about the validity of the data as more than one baseline measurement was presented for each group.

In the study by Romagnoli et al, changes in 1, 25(OH)₂D were reported after a 300 000IU vitamin D₃ bolus in elderly female participants. However, details provided on changes in 1, 25(OH)₂D were extremely brief, only noting that there had been a "sharp increase" in 1, 25(OH)₂D at 3-days post-supplementation, but there were no differences between the treatment groups (oral D₃ vs. oral D₂ vs. IM D₃ vs. IM D₂) for the total AUC (172).

4.6.2 Bolus doses \geq 500 000IU

Cipriani et al also report a sharp increase in 1, 25(OH)₂D 30-days after a larger 600 000IU oral D₃ bolus in vitamin D sufficient healthy older adults (173). The statistically significant increase in 1, 25(OH)₂D from baseline at 30-days (basal difference: +25.5pg/ml [SD: +/- 27] was only present in the oral D₃ administration and not in the other treatment groups (oral D₂ vs. IM D₃ vs. IM D₂), followed by a decrease at 60-, 90- and 120-days (173). The AUC was significantly higher for oral D₃ administration than D₂ (oral D₃: 6833pg/ml, [SD: +/- 84.5] vs. oral D₂: 4879pg/ml, [SD: +/- 83.9], P<. 0001), indicating a greater conversion to the active metabolite after a vitamin D₃ bolus (173).

The same group report a sharp increase 1, 25(OH)₂D levels in a group of vitamin D insufficient younger healthy adults (n=20) just 3-days after a 600 000IU D₃ bolus (214). The basal increase in 1, 25(OH)₂D remained significant up to 60-days post-administration, before returning to baseline levels (Mean: 47pg/ml, [SD: +/- 19]) by 90-days. Mean (SD) basal difference in 1, 25(OH)₂D at 3-, 15-, 30-, 60- and 90-days were 51 (+/- 9), 44 (+/- 9), 28 (+/- 7), 13 (+/- 6), and (6 +/- 5) pg/ml, respectively (214).

Similar findings are reported in another study carried out by the same group. After a 600 000IU D₃ bolus in younger women there was a sharp increase in 1, 25(OH)₂D concentration from baseline at 3-days post administration that remained significantly above baseline up to 30-days (P<. 001). Mean (SD) 1, 25(OH)₂D at baseline, 3-, 15-, 30-, 60-days were 46.9 (+/-19.9), 96.6 (+/- 37.4), 88.1 +/- (44.7), 76.2 (+/-38.3) and 42.2 (+/- 18.8) pg/ml, respectively (313).

4.7 Effect of a single large oral bolus dose of vitamin D₃ on free 25(OH)D

Only one study to date has reported free 25(OH)D levels in response to a single, large bolus dose of vitamin D₃ (245). After administration of a 100 000IU D₃ oral bolus to young healthy participants, calculated free 25(OH)D increased from median baseline values of 6.2pg/ml (range: 3.2-27.9) to 11.1pg/ml (range: 6.2-42.2) at 1-week (245). This approximate 1.8-fold increase was almost identical to the approximate 1.8-fold increase seen in total 25(OH)D (245), suggesting that a 100 000IU dose in young, healthy adults does not saturate of VDBP and cause a disproportionate rise in free 25(OH)D.

Other studies have reported on the response of free 25(OH)D to smaller daily doses of supplementation (314). These studies do demonstrate a dose response increase in free 25(OH)D to vitamin D supplementation and a tight correlation between total 25(OH)D and free 25(OH)D in healthy people (314).

Results

4.8 Response of total 25(OH)D to bolus dose vitamin D₃ (Immunoassay)

4.8.1 Proportional between-group differences at each time point

Total 25(OH)D profiles in response to three different bolus doses of vitamin D₃ supplementation (50 000IU, 150 000IU & 500 000IU) are shown in **figure 28** & **table 16**.

Serum total 25(OH)D increased in a dose-dependent manner, with rapid increases from baseline at week 1 and remained significantly different between treatment groups at week 4 and week 12. There was a statistically significant interaction between treatment group and time point ($P < .001$), after adjustment for baseline total 25(OH)D level, indicating that the difference in total 25(OH)D level between treatment groups changes over time. Peak total 25(OH)D had occurred by week 4 in all treatment groups. The highest achieved total 25(OH)D level by a single participant was 190.3nmol/l, 1 week after administration in the 500 000IU group.

Post-hoc analysis indicated that total 25(OH)D at week 1 was highest in the 500 000IU group compared to the 50 000IU (ratio of difference: 2.95, [95% CI: 2.56, 3.41], $P < .001$) and 150 000IU (ratio of difference = 2.01, [95% CI: 1.74, 2.33], $P < .001$) groups. Total 25(OH)D at week 1 was higher than the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.47, [95% CI: 1.27, 1.70], $P < .001$).

At week 4, total 25(OH)D remained significantly higher in the 500 000IU group compared to the 50 000IU (ratio of difference: 2.47 [95% CI: 2.13, 2.85], $P < .001$) and 150 000IU (ratio of difference: 1.77 [95% CI: 1.54, 2.05], $P < .001$) groups and remained significantly higher in the 150 000IU group vs. 50 000IU (ratio of difference: 1.39 [95% CI: 1.20, 1.61], $P < .001$).

By week 12, total 25(OH)D remained highest in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 2.03 [95% CI: 1.77, 2.35], $P < .001$; difference ratio vs. 150 000IU: 1.57 [95% CI: 1.36, 1.82], $P < .001$) and total 25(OH)D remained higher in the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.29 [95% CI: 1.12, 1.50], $P < .001$). Between-group differences at each time point are summarised in **figure 28**.

4.8.2 Proportional within-group changes

In the 500 000IU treatment group, total 25(OH)D was higher than at baseline in all weeks. At week 1, total 25(OH)D increased sharply from baseline (ratio of difference vs. baseline: 4.53 [95% CI: 3.97, 5.18]) and remained at similar levels at week 4 (ratio of difference vs. baseline: 4.19 [95% CI: 3.67, 4.79]). By week 12, total 25(OH)D level had fallen from weeks 1 and 4 (ratio of difference vs. baseline: 2.89 [95% CI: 2.52, 3.30]).

In the 150 000IU treatment group, total 25(OH)D was higher than at baseline in all weeks. At week 1, total 25(OH)D increased from baseline (ratio of difference vs. baseline: 2.21 [95% CI: 1.92, 2.53]) and remained at similar levels at week 4 (ratio of difference vs. baseline: 2.33 [95% CI: 2.04, 2.66]) and week 12 (ratio of difference vs. baseline: 1.81 [95% CI: 1.58, 2.08]).

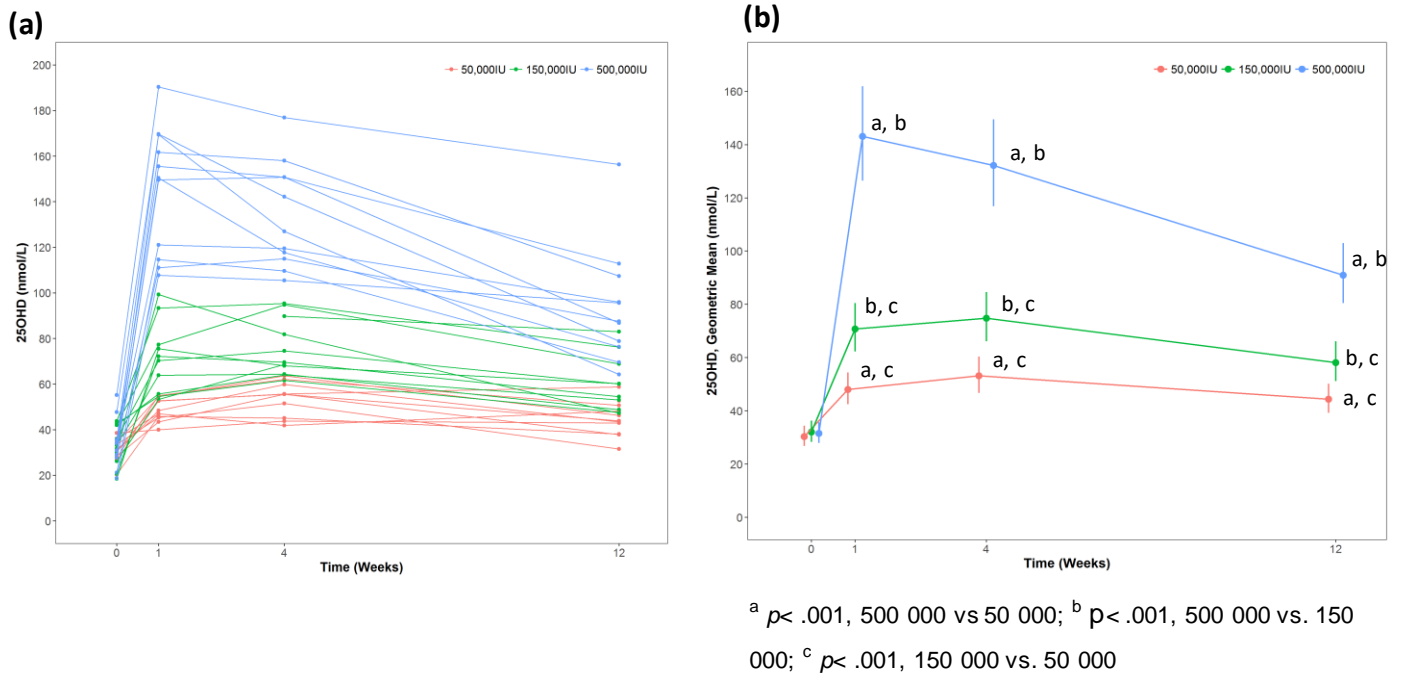
As in the other treatment groups, total 25(OH)D was higher than at baseline in all weeks in the 50 000IU treatment group. At week 1, total 25(OH)D increased from baseline (ratio of difference vs. baseline: 1.58 [95% CI: 1.39, 1.81]) and remained similar at week 4 (ratio of difference vs. baseline: 1.75 [95% CI: 1.52, 2.01]) and week 12 (ratio of difference vs. baseline: 1.46 [95% CI: 1.28, 1.67]). Absolute values at each time point are summarised in **table 16**.

4.8.3 Comparison to control group at week 12

There was a significant difference in total 25(OH)D level between each of the three treatment groups and control group at week 12 (ANOVA, $P < 0.001$). Post-hoc analysis shows that total 25(OH)D level in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.46 [95% CI: 1.18, 1.81], $P = .001$). Total 25(OH)D in the 150 000IU group at week 12 was not significantly different to control levels at week 12 (ratio of difference: 0.94 [95% CI: 0.76, 1.18], $P = .605$). Total 25(OH)D in the 50 000IU group was significantly lower than control levels (ratio of difference: 0.71 [95% CI: 0.58, 0.88], $P = .002$).

Control group levels of total 25(OH)D at baseline (Geometric mean: 67.7nmol/l [95% CI: 54.1, 84.5]) were similar to week 12 (Geometric mean: 62.3nmol/l, 95% CI: 46.6, 83.2). There was no statistically significant difference between the two time points ($t(26) = 1.487$, $P = .149$).

Figure 28: (a) Total 25(OH)D profiles by individual participants in response to the three different single oral bolus doses of vitamin D₃ and; (b) total 25(OH)D by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% Confidence Intervals.

Table 16: Total 25(OH)D (nmol/L) response to bolus dose treatment at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	30.4 (26.8, 34.3)	48.1 (42.5, 54.4)	53.1 (46.7, 60.4)	44.3 (39.2, 50.2)
150 000IU	32.1 (26.9, 38.3)	70.7 (62.2, 80.4)	74.8 (66.1, 84.6)	58.1 (51.1, 66.1)
500 000IU	31.5 (27.9, 35.7)	143.0 (126.4, 161.8)	132.1 (116.8, 149.5)	91.0 (80.4, 103.0)

Values are geometric means and 95% CIs.

4.9 Response of total 25(OH)D₃ to bolus dose supplementation (LC-MS/MS)

4.9.1 Proportional between-group differences at each time point

Total 25(OH)D₃ profiles after treatment followed a similar pattern to total 25(OH)D profiles (**figure 29 & table 17**). A dose-dependent increase in total 25(OH)D₃ was seen, with rapid increases from baseline at week 1 and total 25(OH)D₃ remained significantly different between treatment groups at week 4 and week 12. There was a statistically significant interaction between treatment group and time point ($P < .001$), after adjustment for baseline total 25(OH)D₃ level. Peak total 25(OH)D₃ had occurred by week 1 in all treatment groups. The highest achieved total 25(OH)D₃ level by a single participant was 213.8nmol/l, 1 week after administration in the 500 000IU group.

Post hoc analysis indicated that total 25(OH)D₃ at week 1 was highest in the 500 000IU group compared to the 50 000IU (ratio of difference: 3.26 [95% CI: 2.82, 3.77], $P < .001$) and 150 000IU (ratio of difference: 2.18 [95% CI: 1.87, 2.54], $P < .001$) groups. Total 25(OH)D₃ at week 1 was higher than the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.51 [95% CI: 1.30, 1.76], $P < .001$).

At week 4, total 25(OH)D₃ remained significantly higher in the 500 000IU group compared the 50 000IU (ratio of difference: 2.53 [95% CI: 2.17, 2.95], $P < .001$) and the 150 000IU (ratio of difference: 1.72 [95% CI: 1.48, 2.00], $P < .001$) treatment groups. Total 25(OH)D₃ remained significantly higher in the 150 000IU group vs. 50 000IU (ratio of difference: 1.50 [95% CI: 1.29, 1.76], $P < .001$).

By week 12, total 25(OH)D₃ remained highest in the 500 000IU group compared to the 50 000IU (ratio of difference: 2.32 [95% CI: 2.00, 2.69], $P < .001$) and the 150 000IU (ratio of difference: 1.49 [95% CI: 1.29, 1.73], $P < .001$) treatment groups. Total 25(OH)D₃ remained higher in the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.61 [95% CI: 1.38, 1.87], $P < .001$). Between-group differences at each time point are summarised in **figure 29**.

4.9.2 Proportional within-group changes

In the 500 000IU treatment group, total 25(OH)D₃ was higher than baseline in all weeks. At week 1, total 25(OH)D₃ increased sharply from baseline (ratio of difference vs. baseline: 7.21 [95% CI: 6.34, 8.19]), but had fallen slightly by week 4 (ratio of difference vs. baseline: 5.58 [95% CI: 4.91, 6.34]). By week 12, total 25(OH)D₃ had fallen further from week 4 levels (ratio of difference vs. baseline: 3.63 [95% CI: 3.19, 4.12]).

In the 150 000IU treatment group, total 25(OH)D₃ was higher than baseline in all weeks. At week 1, total 25(OH)D₃ increased sharply from baseline (ratio of difference vs. baseline: 3.40 [95% CI: 2.97, 3.89]) and remained similar at week 4 (ratio of difference vs. baseline 3.38 [95% CI: 2.96, 3.86]). However, by week 12, total 25(OH)D₃ had fallen from levels seen at weeks 1 and 4 (ratio of difference vs. baseline: 2.53 [95% CI: 2.21, 2.89]).

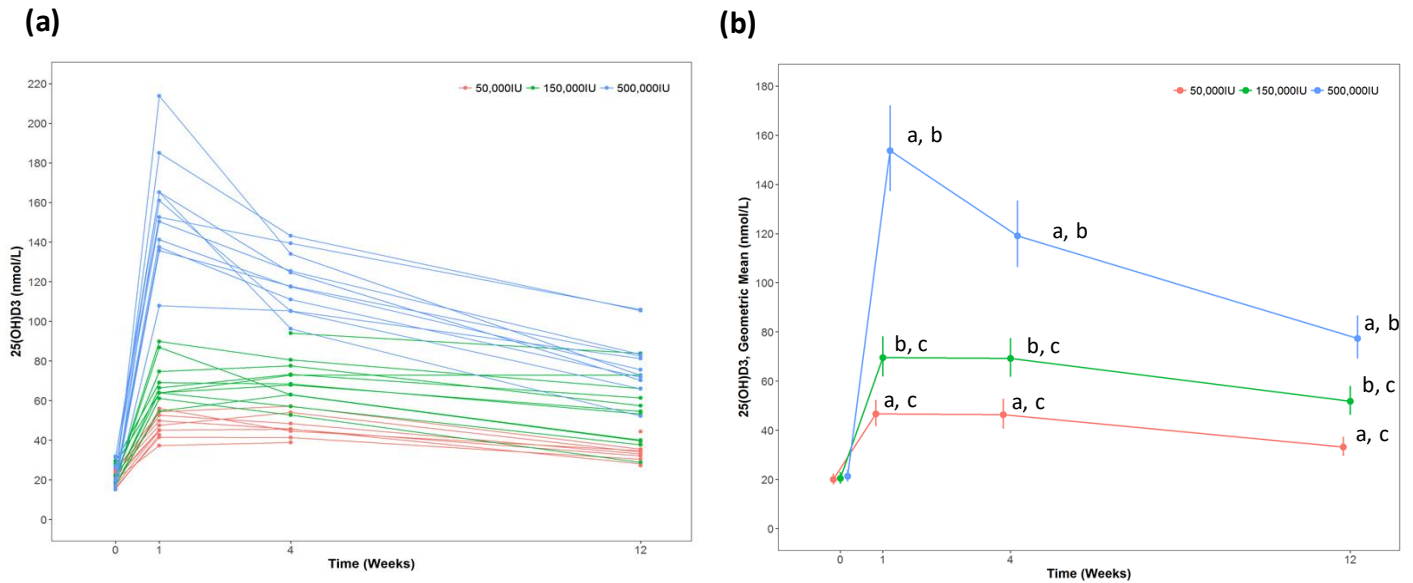
In the 50 000IU treatment group, total 25(OH)D₃ was higher than baseline in all weeks. At week 1, total 25(OH)D₃ increased from baseline (ratio of difference vs. baseline: 2.33 [95% CI: 2.05, 2.65]) and remained similar at week 4 (ratio of difference vs. baseline: 2.31 [95% CI: 2.01, 2.67]). However, by week 12, total 25(OH)D₃ had fallen from levels at weeks 1 and 4 (ratio of difference: 1.66 [95% CI: 1.45, 1.89]). Absolute values at each time point are summarised in **table 17**.

4.9.3 Comparison to control group

There was a significant difference in 25(OH)D₃ levels between each of the three treatment groups and control group at week 12 (ANOVA, $P < 0.001$). Post-hoc analysis shows that mean total 25(OH)D₃ levels in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.53 [95% CI: 1.22, 1.92], $P < .001$). Total 25(OH)D in the 150 000IU group at week 12 was not significantly different to control levels (ratio of difference: 1.03 [95% CI: 0.92, 1.29], $P = .828$). Total 25(OH)D₃ in the 50 000IU group was significantly lower than the control group (ratio of difference: 0.66 [95% CI: 0.52, 0.84], $P < .001$).

Control group levels of total 25(OH)D₃ at baseline (Geometric mean: 55.3nmol/l [95% CI: 44.9, 68.2]) were similar to week 12 (Geometric mean: 50.5nmol/l, 95% CI: 37.1, 68.7). There was no statistically significant difference between the two time points ($t(26) = 1.585$, $P = .125$).

Figure 29: (a) Individual participant profiles for total 25(OH)D₃ in response to bolus dose vitamin D₃ and; (b) 25(OH)D₃ by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 500 000 vs. 150 000; ^c $P < .001$, 150 000 vs. 50 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 17: Total 25(OH)D₃ (nmol/L) response to bolus dose treatment at each time point. Values are geometric means and 95% CIs.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	20.0 (17.9, 22.4)	46.7 (41.7, 52.3)	46.3 (40.7, 52.8)	33.2 (29.5, 37.4)
150 000IU	20.5 (18.2, 23.0)	69.6 (61.8, 78.3)	69.2 (61.8, 77.5)	51.8 (46.2, 58.0)
500 000IU	21.3 (19.1, 23.9)	153.7 (137.3, 172.2)	119.1 (106.3, 133.4)	77.4 (69.1, 86.7)

4.10 Response of 1, 25(OH)₂D to bolus dose vitamin D₃

4.10.1 Proportional between-group differences at each time point

Profiles for change in 1, 25(OH)₂D in response to the three different bolus doses of vitamin D₃ are shown in **figure 30**. **Table 18** shows total 1, 25(OH)₂D values by treatment group and time point. There was a dose-dependent increase in 1, 25(OH)₂D, with rapid increases from baseline at week 1. There was a significant difference in response between treatment groups ($P < .001$), but there was no statistically significant interaction between treatment group and time point ($P = .051$), after adjustment for baseline 1, 25(OH)₂D, suggesting that the difference in 1, 25(OH)₂D level between treatment groups does not change over time.

Post-hoc analysis indicates that 1, 25(OH)₂D was significantly higher in the 500 000IU group compared to the 50 000IU treatment group (ratio of difference: 1.59 [95% CI: 1.33, 1.90], $P < .001$) and the 150 000IU treatment group was significantly higher than the 50 000IU treatment group (ratio of difference: 1.39 [95% CI: 1.17, 1.657], $P < .001$). Between-group differences at each time point are summarised in **figure 30**.

4.10.2 Proportional within-group changes

In the 500 000IU treatment group, 1, 25(OH)₂D was higher than at baseline in all weeks. At week 1, 1, 25(OH)₂D increased sharply from baseline (ratio of difference vs. baseline: 2.28 [95% CI: 1.93, 2.70]). At week 4 (ratio of difference vs. baseline: 1.62 [95% CI: 1.37, 1.92]) and week 12 (ratio of difference vs. baseline: 1.53 [95% CI: 1.29, 1.81]), 1, 25(OH)₂D had fallen but still remained higher than baseline levels (ratio of difference vs. baseline: 1.62 [95% CI: 1.37, 1.92]).

In the 150 000IU treatment group, 1, 25(OH)₂D was higher than at baseline in all weeks. At week 1, 1, 25(OH)₂D increased from baseline (ratio of difference vs. baseline: 1.75 [95% CI: 1.47, 2.08]) and remained at similar levels at week 4 (ratio of difference vs. baseline: 1.74 [95% CI: 1.47, 2.06]). By week 12, 1, 25(OH)₂D had fallen slightly, but was still above baseline (ratio of difference vs. baseline: 1.46 [95% CI: 1.24, 1.73]).

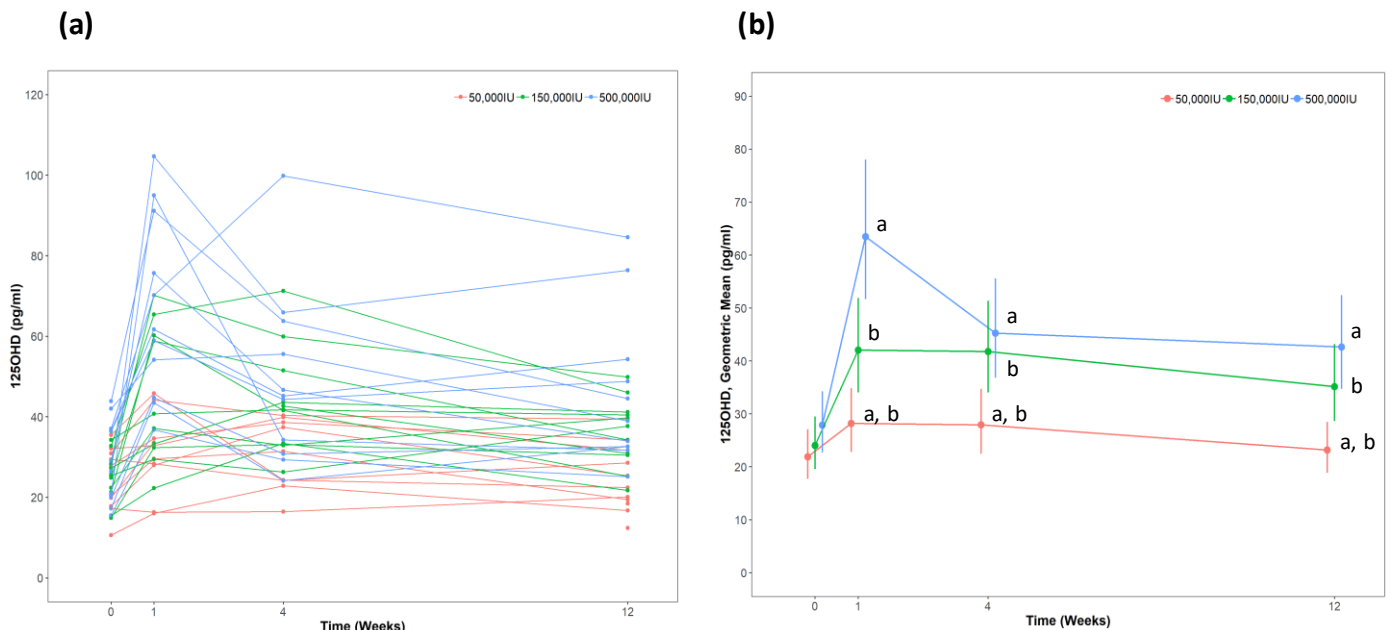
In the 50 000IU treatment group at week 1, 1, 25(OH)₂D increased from baseline (ratio of difference vs. baseline: 1.29 [95% CI: 1.08, 1.53]) and remained similar at week 4 (ratio of difference vs. baseline: 1.27 [95% CI: 1.06, 1.53]). By week 12, 1, 25(OH)₂D was similar to baseline (ratio of difference vs. baseline: 1.05 [95% CI: 0.88, 1.26]). Absolute values at each time point are summarised in **table 18**.

4.10.3 Comparison to control group

There was a significant difference in $1, 25(\text{OH})_2\text{D}$ between each of the three treatment groups and control group at week 12 (ANOVA, $P < 0.001$). Post-hoc analysis shows that $1, 25(\text{OH})_2\text{D}$ level in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.34 [95% CI: 1.07, 1.70], $P = .014$). There was no significant difference in circulating $1, 25(\text{OH})_2\text{D}$ between the 150 000IU group and control group at week 12 (ratio of difference: 1.11 [95% CI: 0.88, 1.40], $P = .378$), but $1, 25(\text{OH})_2\text{D}$ in the 50 000IU group was significantly lower than control levels (ratio of difference: 0.73 [95% CI: 0.58, 0.92], $P = .009$).

Control group levels of $1, 25(\text{OH})_2\text{D}$ at baseline (Geometric mean: 33.4pg/ml [95% CI: 25.1, 44.4]) were similar to week 12 (Geometric mean: 31.7pg/ml, 95% CI: 24.6, 40.8). There was no statistically significant difference between the two time points ($t(26) = 1.041$, $P = .308$).

Figure 30: (a) Individual participant profiles for $1, 25(\text{OH})_2\text{D}$ in response to bolus vitamin D_3 and; (b) $1, 25(\text{OH})_2\text{D}$ response by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 150 000 vs. 50 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 18: Total 1, 25(OH)₂D (pg/ml) response to bolus dose treatment at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	21.9 (17.7, 27.1)	28.1 (22.9, 34.8)	27.9 (22.5, 34.7)	23.2 (18.9, 28.5)
150 000IU	24.0 (19.5, 30.0)	42.0 (34.0, 51.9)	41.8 (34.0, 51.3)	35.1 (28.6, 41.2)
500 000IU	27.9 (22.7, 34.2)	63.5 (51.7, 78.0)	45.2 (36.8, 55.6)	42.7 (34.7, 52.4)

Values are geometric means and 95% CIs.

4.11 Response of measured free 25(OH)D to bolus dose supplementation

4.11.1 Proportional between-group differences at each time point

Measured free 25(OH)D profiles in response to three different bolus doses of cholecalciferol supplementation are shown in **figure 31 & table 19**.

Like total 25(OH)D and total 25(OH)D₃, measured free increased in a dose-dependent manner, with rapid increases from baseline at week 1 and remained significantly different between treatment groups at week 4 and week 12. There was a statistically significant interaction between treatment group and time point ($P < 0.001$), after adjustment for baseline measured free 25(OH)D level. Peak measured free 25(OH)D occurred by week 4 in all treatment groups.

Post-hoc analysis indicated that measured free at week 1 was highest in the 500 000IU group compared to the 50 000IU (ratio of difference: 3.74 [95% CI: 3.19, 4.29], $P < .001$) and 150 000IU (ratio of difference: 2.56 [95% CI: 2.18, 3.02], $P < .001$) treatment groups. Measured free 25(OH)D at week 1 was higher in the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.46 [95% CI: 1.24, 1.72], $P < .001$).

At week 4, measured free 25(OH)D remained significantly higher in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 2.50 [95% CI: 2.13, 2.94], $P < .001$; ratio of difference vs. 150 000IU: 1.70 [95% CI: 1.44, 2.00], $P < .001$). Measured free remained significantly higher in the 150 000IU group compared to the 50 000IU treatment group (difference ratio: 1.47, [95% CI: 1.25, 1.74], $P < .001$).

By week 12, measured free 25(OH)D remained highest in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 1.97 [95% CI: 1.68, 2.31], $P < .001$; difference ratio vs. 150 000IU: 1.39, [95% CI: 1.18, 1.63], $P < .001$). Measured free 25(OH)D remained higher in the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.42 [95% CI: 1.21, 1.67], $P < .001$). Between-group differences at each time point are summarised in **figure 31**.

4.11.2 Proportional within-group changes

In the 500 000IU treatment group, measured free 25(OH)D was higher than baseline in all weeks. At week 1, measured free 25(OH)D increased sharply from baseline (ratio of difference vs. baseline: 7.09 [95% CI: 5.73, 8.88]). Interestingly, this proportional increase is similar to the proportional change seen in total 25(OH)D and total 25(OH)D₃ in this treatment group. Levels of measured free 25(OH)D remained similar at week 4 (ratio of difference vs. baseline: 5.17 [95% CI: 4.18, 6.40]). By week 12, measured free 25(OH)D had fallen from week 1 and week 4 levels (ratio of difference vs. baseline: 3.28 [95% CI: 3.19, 4.12]). The proportional changes from baseline in measured free 25(OH)D at weeks 4 and 12 were of similar magnitude to the proportional changes seen in total 25(OH)D and total 25(OH)D₃ at these time points in the 500 000IU treatment group.

In the 150 000IU treatment group, measured free 25(OH)D was higher than baseline in all weeks. At week 1, measured free 25(OH)D increased sharply from baseline (ratio of difference vs. baseline: 2.07 [95% CI: 1.66, 2.61]) and remained similar at week 4 (ratio of difference vs. baseline: 2.47 [95% CI: 1.98, 3.07]) and week 12 (ratio of difference vs. baseline: 2.03 [95% CI: 1.63, 2.52]). The proportional changes from baseline in measured free 25(OH)D at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in total 25(OH)D and total 25(OH)D₃ at these time points in the 150 000IU treatment group.

In the 50 000IU treatment group, measured free 25(OH)D was higher than baseline in all weeks. At week 1, measured free 25(OH)D increased from baseline (ratio of difference vs. baseline: 1.72 [95% CI: 1.41, 2.11]) and remained similar at week 4 (ratio of difference vs. baseline: 1.88 [95% CI: 1.41, 2.11]) and week 12 (ratio of difference vs. baseline: 1.51 [95% CI: 1.22, 1.87]). The proportional changes from

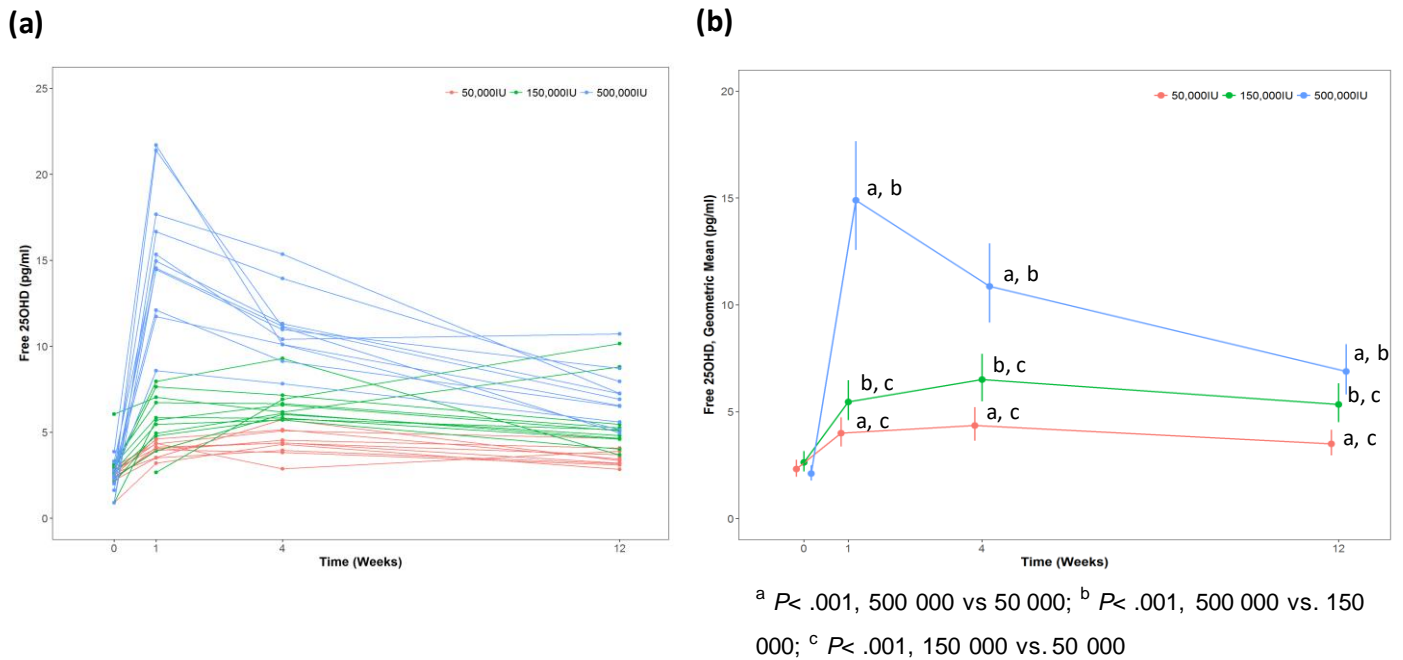
baseline in measured free 25(OH)D at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in total 25(OH)D and total 25(OH)D₃ at these time points in the 50 000IU treatment group. Absolute values at each time point are summarised in **table 19**.

4.11.3 Comparison to control group

There was a significant difference in measured free 25(OH)D between the three treatment groups and control group at week 12 (ANOVA, $P < .001$). Post-hoc analysis shows that measured free 25(OH)D level in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.57 [95% CI: 1.26, 1.96], $P < .001$). Measured free 25(OH)D in the 150 000IU group at week 12 was not significantly different to control levels at week 12 (ratio of difference: 1.22 [95% CI: 0.98, 1.52], $P = .076$). Measured free 25(OH)D in the 50 000IU group was significantly lower than control levels (ratio of difference: 0.80 [95% CI: 0.64, 0.99], $P < .001$).

Control group levels of measured free 25(OH)D at baseline (Geometric mean: 5.2pg/ml [95% CI: 4.2, 6.3]) was higher than at week 12 (Geometric mean: 4.36pg/ml [95% CI: 3.25, 5.9]). There was a statistically significant difference between the two time points ($t(26) = 2.373$, $P = .025$).

Figure 31: (a) Individual participant profiles for measured free 25(OH)D in response to a single oral bolus dose of vitamin D₃ and; (b) measured free 25(OH)D response by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 19: Measured free 25(OH)D (pg/ml) response to bolus dose treatment at each time point. Values are geometric means and 95% CIs.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	2.3 (2.0, 2.7)	4.0 (3.7, 4.7)	4.4 (3.6, 5.2)	3.5 (3.0, 4.2)
150 000IU	2.6 (2.2, 3.2)	5.5 (4.6, 6.5)	6.5 (5.5, 7.7)	5.3 (4.5, 6.3)
500 000IU	2.1 (1.8, 2.5)	14.9 (12.6, 17.7)	10.9 (9.2, 12.9)	6.9 (5.8, 8.2)

4.12 Change in calculated free 25(OH)D (using total 25(OH)D measured by immunoassay) in response to bolus dose vitamin D₃

4.12.1 Proportional between-group differences at each time point

Calculated free 25(OH)D profiles after treatment were similar to measured free 25(OH)D. Profiles are shown in **figure 32**. Calculated free 25(OH)D also increased in a dose-dependent manner. There was a statistically significant interaction between treatment group and time point ($P = .005$), after adjustment for baseline calculated free 25(OH)D level. Peak calculated free 25(OH)D occurred by week 4 in all treatment groups.

Post hoc analysis indicated that calculated free 25(OH)D at week 1 was highest in the 500 000IU group compared to the 50 000IU (ratio of difference vs. baseline: 2.88, [95% CI: 2.41, 3.45], $P < .001$) and 150 000IU (ratio of difference vs. baseline: 1.93, [95% CI: 1.61, 2.32], $P < .001$) groups. Calculated free 25(OH)D at week 1 was higher in the 150 000IU group compared to the 50 000IU group (ratio of difference vs. baseline: 1.49, [95% CI: 1.24, 1.80], $P < .001$).

At week 4, calculated free 25(OH)D remained significantly higher in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 2.71, [95% CI: 2.25, 3.25], $P < .001$; ratio of difference vs. 150 000IU: 1.76, [95% CI: 1.47, 2.11], $P < .001$). Calculated free 25(OH)D also remained significantly higher in the 150 000IU group vs. 50 000IU (difference ratio: 1.53, [95% CI: 1.28, 1.85], $P < .001$).

By week 12, calculated free 25(OH)D remained highest in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 1.94, [95% CI: 1.62, 2.33], $P < .001$; ratio of difference vs. 150 000IU: 1.67, [95% CI: 1.39, 2.00], $P < .001$). By week 12, calculated free 25(OH)D was not significantly different in the 150 000IU group compared to the 50 000IU group (difference ratio: 1.17, [95% CI: 0.97, 1.40], $P = .102$). Between-group differences at each time point are summarised in **figure 32**.

4.12.2 Proportional within-group changes

In the 500 000IU treatment group, calculated free 25(OH)D was higher than baseline in all weeks. At week 1, calculated free 25(OH)D increased sharply from baseline (ratio of difference vs. baseline: 4.36 [95% CI: 3.67, 5.18]). Like measured free 25(OH)D, this proportional change from baseline is similar to the proportional change seen in total 25(OH)D and total 25(OH)D₃ in this treatment group at week 1. Levels of calculated free 25(OH)D remained similar at week 4 (ratio of difference vs. baseline: 4.32 [95%

CI: 3.64, 5.13]). By week 12, calculated free 25(OH)D had fallen from week 1 and week 4 levels (ratio of difference vs. baseline: 2.97 [95% CI: 2.5, 2.97]). The proportional changes from baseline in calculated free 25(OH)D at weeks 4 and 12 were of similar magnitude to the proportional changes seen in measured free 25(OH)D and other vitamin D metabolites at these time points in the 500 000IU treatment group.

In the 150 000IU treatment group, calculated free 25(OH)D was higher than baseline in all weeks. At week 1, calculated free 25(OH)D increased from baseline (ratio of difference vs. baseline: 2.25 [95% CI: 1.88, 2.68]) and remained similar at week 4 (ratio of difference vs. baseline: 2.44 [95% CI: 2.06, 2.90]) and week 12 (ratio of difference vs. baseline: 1.77 [95% CI: 1.48, 2.12]). The proportional changes from baseline in calculated free 25(OH)D at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in measured free 25(OH)D (and total 25(OH)D and total 25(OH)D₃) at these time points in the 150 000IU treatment group.

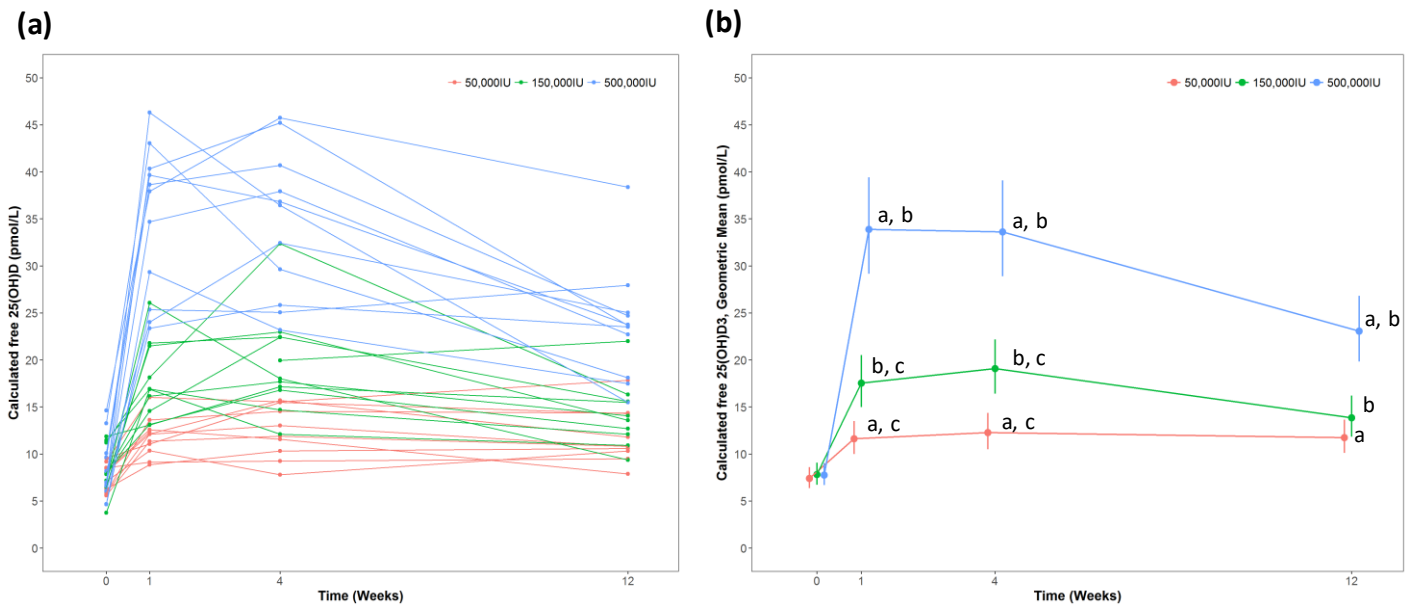
In the 50 000IU treatment group, calculated free 25(OH)D was higher than baseline in all weeks. At week 1, calculated free 25(OH)D increased from baseline (ratio of difference vs. baseline: 1.57 [95% CI: 1.32, 1.87]) and remained similar at week 4 (ratio of difference vs. baseline: 1.67 [95% CI: 1.39, 1.98]) and week 12 (ratio of difference vs. baseline: 1.59 [95% CI: 1.34, 1.89]). The proportional changes from baseline in calculated free 25(OH)D at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in measured free 25(OH)D (and total 25(OH)D and total 25(OH)D₃) at these time points in the 50 000IU treatment group. Absolute values at each time point are summarised in **table 20**.

4.12.3 Comparison to control group

There was a significant difference in calculated free 25(OH)D levels between each of the three treatment groups and control group at week 12 (ANOVA, $P < .001$). Post-hoc analysis shows that calculated free 25(OH)D levels in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.52 [95% CI: 1.18, 1.94], $P < .001$). There was no significant difference in calculated free 25(OH)D at week 12 between the 150 000IU group and control group (ratio of difference: 0.91 [95% CI: 0.71, 1.18], $P = .475$). Calculated free 25(OH)D in the 50 000IU group was significantly lower than the control level (ratio of difference: 0.77 [95% CI: 0.60, 0.99], $P = .042$).

Control group levels of calculated free 25(OH)D at baseline (Geometric mean: 16.6pmol/l [95% CI: 12.8, 21.6]) were similar to week 12 (Geometric mean: 15.2pmol/l [95% CI: 10.8, 21.5]). There was no statistically significant difference between the two time points ($t(26) = 1.485$, $P = .150$).

Figure 32: (a) Individual participant profiles for calculated free 25(OH)D in response to a single oral bolus dose of vitamin D₃ and; (b) calculated free 25(OH)D response by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 500 000 vs. 150 000;
^c $P < .001$, 150 000 vs. 50 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 20: Calculated free 25(OH)D (pmol/L) response to bolus dose treatment at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	7.4 (6.4, 8.6)	11.6 (10.0, 13.5)	12.2 (10.5, 14.4)	11.7 (10.1, 13.7)
150 000IU	7.8 (6.7, 9.1)	17.5 (15.0, 20.5)	19.1 (16.4, 22.2)	13.9 (11.8, 16.2)
500 000IU	7.8 (6.7, 9.0)	33.9 (29.1, 39.4)	33.6 (28.9, 39.1)	23.1 (19.8, 26.8)

Values are geometric means and 95% CIs

4.13 Change in calculated free 25(OH)D₃ (using total 25(OH)D₃ measured by LCMS-MS) in response to bolus dose vitamin D₃

4.13.1 Proportional between-group differences at each time point

Calculated free 25(OH)D₃ profiles after treatment were similar to calculated 25(OH)D and measured free 25(OH)D. Calculated free 25(OH)D₃ responded to supplementation in a dose-dependent manner.

Profiles for calculated free 25(OH)D₃ are shown in **figure 33**. There was a statistically significant interaction between treatment group and time point ($P < .001$), after adjustment for baseline calculated free 25(OH)D₃ level. Peak calculated free 25(OH)D₃ occurred by week 4 in all treatment groups.

Post hoc analysis indicated that calculated free 25(OH)D₃ at week 1 was highest in the 500 000IU group compared to the 50 000IU (ratio of difference: 3.15, [95% CI: 2.62, 3.79], $P < .001$) and 150 000IU (ratio of difference: 2.10, [95% CI: 1.61, 2.32], $P < .001$) groups. Calculated free 25(OH)D₃ at week 1 was higher in the 150 000IU group compared to the 50 000IU group (ratio of difference: 1.50, [95% CI: 1.23, 1.82], $P < .001$).

At week 4, calculated free 25(OH)D₃ remained significantly higher in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 2.72, [95% CI: 2.23, 3.32], $P < .001$; difference ratio vs. 150 000IU: 1.64, [95% CI: 1.36, 1.98], $P < .001$). Calculated free 25(OH)D₃ also remained significantly higher in the 150 000IU group compared to the 50 000IU (ratio of difference: 1.66, [95% CI: 1.35, 2.03], $P < .001$).

By week 12, calculated free 25(OH)D₃ remained highest in the 500 000IU group compared to the other treatment groups (ratio of difference vs. 50 000IU: 1.43, [95% CI: 1.78, 2.60], $P < .001$; ratio of difference vs. 150 000IU: 1.50, [95% CI: 1.24, 1.81], $P < .001$). Calculated free 25(OH)D₃ was still significantly higher in the 150 000IU group compared to the 50 000IU group (difference ratio: 1.22, [95% CI: 1.18, 1.74], $P < .001$). Between-group differences at each time point are summarised in **figure 33**.

4.13.2 Proportional within-group changes

In the 500 000IU treatment group, calculated free 25(OH)D₃ was higher than at baseline in all weeks. At week 1, calculated free 25(OH)D₃ increased sharply from baseline (ratio of difference vs. baseline: 6.93 [95% CI: 5.88, 8.16]) and remained similar at week 4 (ratio of difference vs. baseline: 5.76 [95% CI: 4.89, 6.79]). By week 12, calculated free 25(OH)D₃ had fallen from week 1 and week 4 levels (ratio of difference vs. baseline: 3.73 [95% CI: 3.16, 4.39]). The proportional changes from baseline in calculated free 25(OH)D₃ at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in

measured free 25(OH)D and other vitamin D metabolites at these time points in the 500 000IU treatment group.

In the 150 000IU treatment group, calculated free 25(OH)D₃ was higher than baseline in all weeks. At week 1, calculated free 25(OH)D₃ increased from baseline (ratio of difference vs. baseline: 3.49 [95% CI: 2.93, 4.15]) and remained similar at week 4 (ratio of difference vs. baseline: 3.57 [95% CI: 3.01, 4.22]). By week 12, absolute calculated free 25(OH)D₃ values had fallen slightly from weeks 1 and 4 and the proportional change from baseline was lower than week 4 (ratio of difference vs. baseline: 2.52 [95% CI: 2.13, 2.99]). These proportional changes from baseline in calculated free 25(OH)D₃ at weeks 1, 4 and 12 were of similar magnitude to the proportional changes seen in calculated free 25(OH)D and measured free 25(OH)D (and total 25(OH)D and total 25(OH)D₃) at these time points in the 150 000IU treatment group.

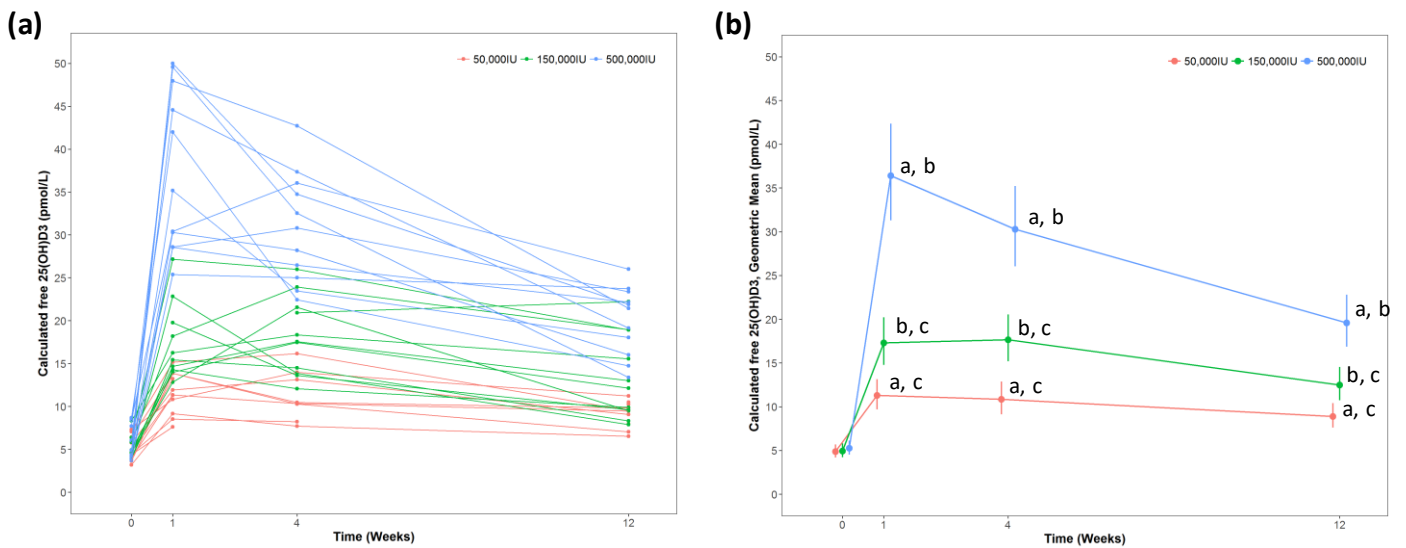
In the 50 000IU treatment group, calculated free 25(OH)D₃ was also higher than baseline in all weeks. At week 1, calculated free 25(OH)D₃ increased from baseline (ratio of difference vs. baseline: 2.31 [95% CI: 1.96, 2.73]) and remained similar at week 4 (ratio of difference vs. baseline: 2.22 [95% CI: 1.86, 2.67]) and week 12 (ratio of difference vs. baseline: 1.82 [95% CI: 1.54, 2.16]). These proportional changes from baseline were again of similar magnitude to the proportional changes seen in calculated free 25(OH)D and measured free 25(OH)D (and total 25(OH)D and total 25(OH)D₃) at these time points in the 50 000IU treatment group. Absolute values at each time point are summarised in **table 21**.

4.13.3 Comparison to control group

There was a significant difference in calculated free 25(OH)D₃ levels between each of the three treatment groups and control group at week 12 (ANOVA, $P < .001$). Post-hoc analysis shows that calculated free 25(OH)D₃ levels in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.59 [95% CI: 1.22, 2.07], $P < .001$). There was no significant difference in calculated free 25(OH)D₃ between the 150 000IU group and control group at week 12 (ratio of difference: 0.78 [95% CI: 0.78, 1.32], $P = .917$), but calculated 25(OH)D₃ in the 50 000IU group was significantly lower than the control level (ratio of difference: 0.73 [95% CI: 0.56, 0.96], $P = .025$).

Control group levels of calculated free 25(OH)D₃ at baseline (Geometric mean: 13.6pmol/l [95% CI: 10.8, 17.0]) were similar to week 12 (Geometric mean: 12.33pmol/l [95% CI: 8.6, 17.6]). There was no statistically significant difference between the two time points ($t(26) = 1.621$, $P = .117$).

Figure 33: (a) Individual participant profiles for calculated free 25(OH)D₃ in response to oral bolus vitamin D₃ and; (b) calculated free 25(OH)D₃ response by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 500 000 vs. 150 000;
^c $P < .001$, 150 000 vs. 50 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 21: Calculated free 25(OH)D₃ (pmol/L) response to bolus dose treatment at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	4.9 (4.2, 5.7)	11.3 (9.7, 13.1)	10.9 (9.1, 12.9)	8.9 (7.6, 10.4)
150 000IU	5.0 (4.2, 5.8)	17.3 (14.8, 20.2)	17.7 (15.2, 20.5)	12.5 (10.7, 14.5)
500 000IU	5.3 (4.5, 6.1)	36.4 (31.3, 42.4)	30.3 (26.0, 35.2)	19.6 (16.8, 22.8)

Values are geometric means and 95% CIs.

4.14 Change in calculated free 1, 25(OH)₂D in response to bolus dose vitamin D₃

4.14.1 Proportional between-group difference at each time point

Profiles for change in calculated free 1, 25(OH)₂D in response to the three different bolus doses of cholecalciferol are shown in **figure 34**. There was a dose-dependent response in calculated free 1, 25(OH)₂D, with increases in calculated free 1, 25(OH)₂D from baseline at week 1. There was no statistically significant interaction between treatment group and time point ($P = .197$), after adjustment for baseline calculated free 1, 25(OH)₂D, suggesting that the difference in 1, 25(OH)₂D level between treatment groups does not change over time. There was a significant difference in response between treatment groups ($P < .001$).

Post-hoc analysis indicates that calculated free 1, 25(OH)₂D was significantly higher in the 500 000IU group compared to the 50 000IU treatment group (ratio of difference: 1.59 [95% CI: 1.29, 1.96], $P < .001$) and the 150 000IU treatment group was significantly higher than the 50 000IU treatment group (ratio of difference: 1.41 [95% CI: 1.15, 1.73], $P < .001$). Between-group differences at each time point are summarised in **figure 34**.

4.14.2 Proportional within-group changes

In the 500 000IU treatment group, calculated free 1, 25(OH)₂D was higher than at baseline in all weeks. At week 1, calculated free 1, 25(OH)₂D increased from baseline (ratio of difference vs. baseline: 2.19 [95% CI: 1.81, 2.65]) and remained similar at week 4 (ratio of difference vs. baseline: 1.67 [95% CI: 1.38, 2.03]) and week 12 (ratio of difference vs. baseline: 1.57 [95% CI: 1.30, 1.90]).

In the 150 000IU treatment group, calculated free 1, 25(OH)₂D was higher than at baseline in all weeks. At week 1, calculated free 1, 25(OH)₂D increased from baseline (ratio of difference vs. baseline: 1.78 [95% CI: 1.46, 2.16]) and remained at similar levels at week 4 (ratio of difference vs. baseline: 1.82 [95% CI: 1.50, 2.20]) and week 12 (ratio of difference vs. baseline: 1.45 [95% CI: 1.20, 1.75]).

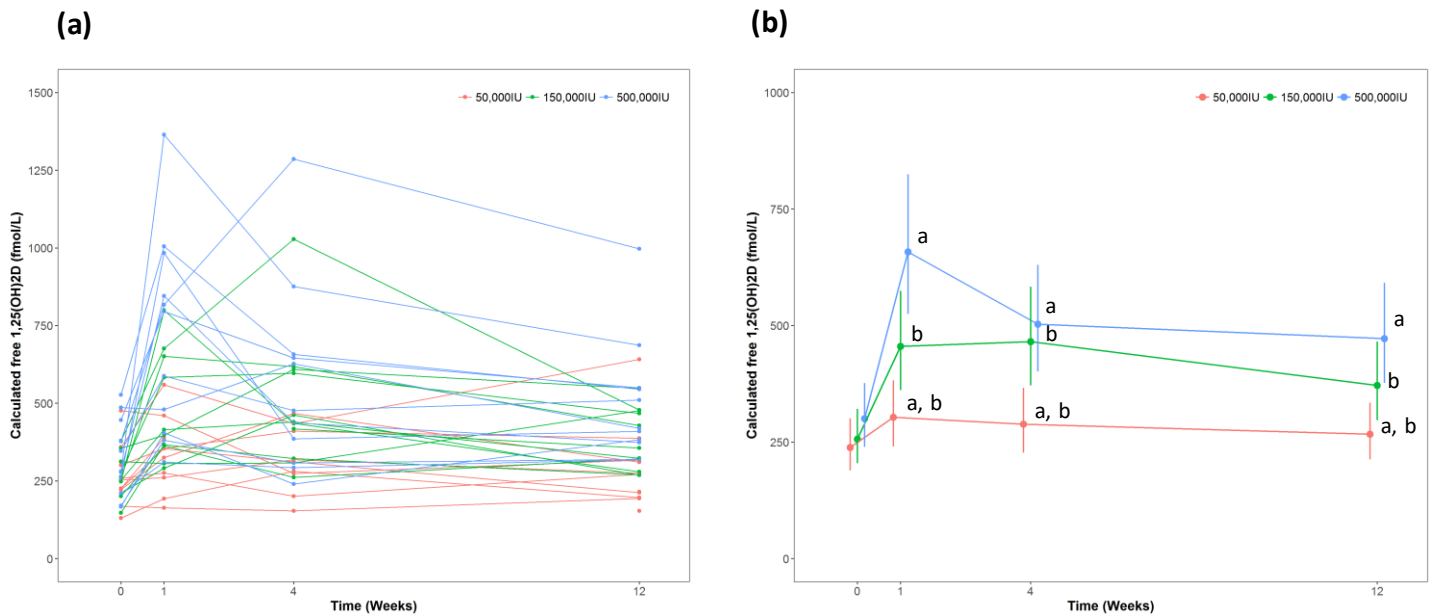
In the 50 000IU treatment group, calculated free 1, 25(OH)₂D was higher than at baseline in all weeks. At week 1, calculated free 1, 25(OH)₂D increased from baseline (ratio of difference vs. baseline: 1.27 [95% CI: 1.04, 1.55]) and remained similar at week 4 (ratio of difference vs. baseline: 1.21 [95% CI: 0.98, 1.49]) and week 12 (ratio of difference vs. baseline: 1.12 [95% CI: 0.92, 1.36]). Absolute values at each time point are summarised in **table 22**.

4.14.3 Comparison to control group

There was a significant difference in calculated 1, 25(OH)₂D between the three treatment groups and control group at week 12 (ANOVA, $P = .003$). Post-hoc analysis shows that calculated free 1, 25(OH)₂D in the 500 000IU group remained significantly higher than the control group (ratio of difference: 1.39 [95% CI: 1.09, 1.78], $P < .001$). Calculated free 1, 25(OH)₂D in the 150 000IU group (ratio of difference: 1.10 [95% CI: 0.86, 1.40], $P = .456$) and 50 000IU group (ratio of difference: 0.78 [95% CI: 0.62, 1.01], $P = .056$) was not significantly different to control levels.

Control group levels of calculated free 1, 25(OH)D at baseline (Geometric mean: 357 fmol/l [95% CI: 268, 476]) were similar to week 12 (Geometric mean: 338 fmol/l [95% CI: 257, 447]). There was no statistically significant difference between the two time points ($t(26) = 1.037$, $P = .309$).

Figure 34: (a) Individual participant profiles for calculated free 1, 25(OH)D in response to bolus dose of vitamin D₃ and; (b) calculated free 1, 25(OH)D by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 150 000 vs. 50 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 22: Calculated free 1, 25(OH)₂D (fmol/l) response to bolus dose treatment at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	238 (189, 301)	303 (240, 383)	289 (227, 367)	267 (213, 334)
150 000IU	256 (204, 321)	456 (362, 574)	465 (371, 583)	372 (297, 466)
500 000IU	300 (240, 376)	658 (525, 824)	503 (401, 630)	472 (377, 592)

Values are geometric means and bars represent 95% CIs.

4.15 Change in percent measured free 25(OH)D (measured free 25(OH)D: total 25(OH)D) in response to bolus dose vitamin D₃

4.15.1 Proportional between-group changes across time points

Change in the percent measured free 25(OH)D are shown in **figure 35** and **table 23**. There was a statistically significant interaction between treatment group and time point ($P = .002$).

Post hoc analysis indicated that the proportional increase in percent measured free 25(OH)D at week 1 was significantly higher in the 500 000IU group compared to the 50 000IU (ratio of difference: 1.26, [95% CI: 1.04, 1.53], $P = .020$) and 150 000IU (ratio of difference: 1.25, [95% CI: 1.03, 1.53], $P = .028$) groups. The percent measured free 25(OH)D at week 1 in the 150 000IU group was not significantly different to the 50 000IU group (ratio of difference: 1.01, [95% CI: 0.83, 1.23], $P = .935$).

At week 4, the percent measured free 25(OH)D was not significantly different between each treatment group (ratio of difference 500 000IU vs. 50 000IU: 1.01, [95% CI: 0.87, 1.28], $P = .940$; ratio of difference 500 000 vs. 150 000IU: 0.96, [95% CI: 0.79, 1.17], $P = .661$; ratio of difference 150 000IU vs. 50 000IU: 1.05, [95% CI: 0.87, 1.16], $P = .607$).

The percent measured free 25(OH)D at week 12 was also not significantly different between each treatment groups (ratio of difference 500 000IU vs. 50 000IU: 0.96, [95% CI: 0.79, 1.17], $P = .699$; ratio of difference 500 000 vs. 150 000IU: 0.83, [95% CI: 0.82, 1.08], $P = .065$; ratio of difference 150 000IU vs. 50 000IU: 1.16, [95% CI: 0.96, 1.41], $P = .132$). Between-group differences at each time point are summarised in **figure 35**.

4.15.2 Proportional within-group changes across time points

In the 500 000IU treatment group at week 1, the percent measured free 25(OH)D increased slightly from baseline (ratio of difference vs. baseline: 1.56 [95% CI: 1.26, 1.94]). At week 4 the percent measured free 25(OH)D was similar to baseline (ratio of difference vs. baseline: 1.23 [95% CI: 1.00, 1.53]) and remained similar to baseline at week 12 (ratio of difference vs. baseline: 1.14 [95% CI: 0.92, 1.41]).

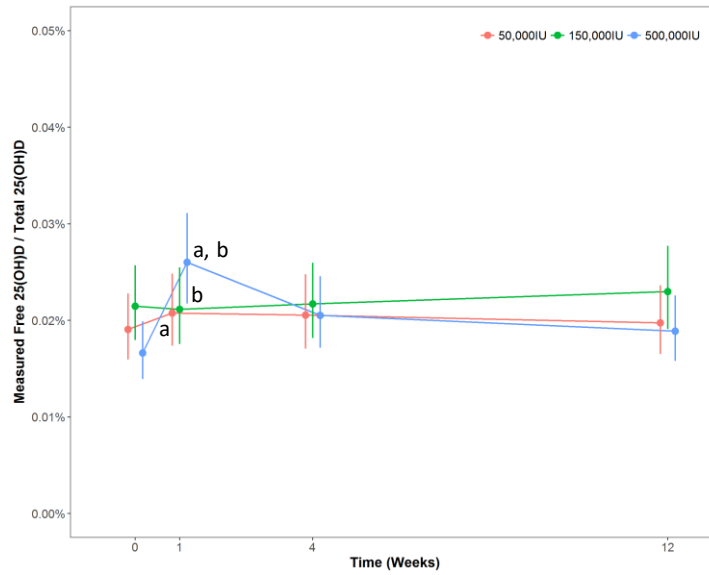
In the 150 000IU treatment group, the percent measured free 25(OH)D remained similar to baseline at all weeks (ratio of difference week 1 vs. baseline: 0.98 [95% CI: 0.79, 1.23]; ratio of difference week 4 vs. baseline: 1.01 [95% CI: 0.82, 1.25]; ratio of difference week 12 vs. baseline: 1.07 [95% CI: 0.86, 1.34]).

The percent measured free 25(OH)D also remained similar to baseline at all weeks in the 50 000IU group (ratio of difference week 1 vs. baseline: 1.09 [95% CI: 0.88, 1.35]; ratio of difference week 4 vs. baseline: 1.08 [95% CI: 0.86, 1.35]; ratio of difference week 12 vs. baseline: 1.04 [95% CI: 0.84, 1.28]). Absolute percent measured free 25(OH)D vales at each time point are summarised in **table 23**.

4.15.3 Comparison to control group

There was a significant difference in the percent measured free 25(OH)D between each of the three treatment groups and control group at week 12 (ANOVA, $P = .044$). Post-hoc analysis shows that the percent measured free 25(OH)D was not significantly different to the control group in the 500 000IU group (ratio of difference: 1.08 [95% CI: 0.90, 1.29], $P = .421$). The percent measured free 25(OH)D in the 150 000IU group was significantly higher than the control group (ratio of difference: 1.31 [95% CI: 1.09, 1.58], $P = .005$). The percent measured free 25(OH)D was similar to the control group in the 50 000IU treatment group (ratio of difference: 1.12 [95% CI: 0.94, 1.35], $P = .198$).

Figure 35: Percentage measured free 25(OH)D (measured free 25(OH)D/ total 25(OH)D) by treatment group.



^a $P < .001$, 500 000 vs 50 000; ^b $P < .001$, 500 000 vs. 150 000

Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 23: Percent measured free 25(OH)D (measured free 25(OH)D/ total 25(OH)D) by treatment group at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.019 (0.016, 0.023)	0.021 (0.017, 0.025)	0.021 (0.017, 0.025)	0.020 (0.016, 0.024)
150 000IU	0.021 (0.018, 0.026)	0.021 (0.015, 0.025)	0.022 (0.018, 0.026)	0.023 (0.019, 0.028)
500 000IU	0.017 (0.014, 0.020)	0.026 (0.022, 0.031)	0.021 (0.017, 0.025)	0.019 (0.016, 0.023)

Values are geometric means and bars represent 95% CIs

4.16 Change in percent calculated free 25(OH)D (calculated free 25(OH)D: total 25(OH)D) in response to bolus dose supplementation

4.16.1 Proportional between-group changes across time points

Change in the percent calculated free 25(OH)D are shown in **figure 36** and **table 24**. There was no statistically significant interaction between treatment group and time point ($P = .081$). There was no overall statistically significant difference between groups ($P = .986$).

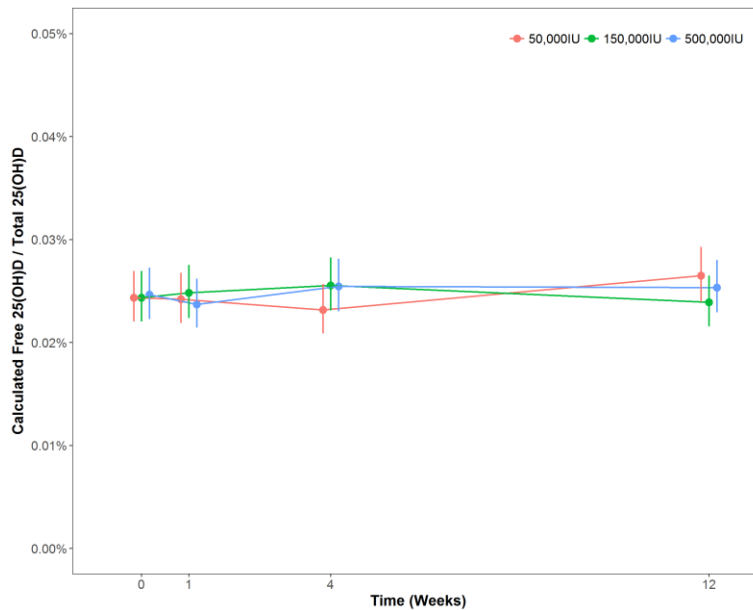
4.16.2 Proportional within-group changes across time points

In the 500 000IU treatment group the percent calculated free 25(OH)D did not change over time (ratio of difference week 1 vs. baseline: 0.96 [95% CI: 0.88, 1.06]; ratio of difference week 4 vs. baseline: 1.03 [95% CI: 0.94, 1.13]; ratio of difference week 12 vs. baseline: 1.03 [95% CI: 0.94, 1.13]).

In the 150 000IU treatment group, the percent calculated free 25(OH)D also remained similar to baseline at all weeks (ratio of difference week 1 vs. baseline: 1.02 [95% CI: 0.92, 1.12]; ratio of difference week 4 vs. baseline: 1.05 [95% CI: 0.95, 1.15]; ratio of difference week 12 vs. baseline: 0.98 [95% CI: 0.89, 1.08]).

The percent calculated free 25(OH)D also remained similar to baseline at all weeks in the 50 000IU group (ratio of difference week 1 vs. baseline: 0.99 [95% CI: 0.90, 1.09]; ratio of difference week 4 vs. baseline: 0.95 [95% CI: 0.86, 1.05]; ratio of difference week 12 vs. baseline: 1.09 [95% CI: 0.99, 1.19]). Absolute percent calculated free 25(OH)D vales at each time point are summarised in **table 24**.

Figure 36: Percentage calculated free 25(OH)D (calculated free 25(OH)D/ total 25(OH)D) by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 24: Percent calculated free 25(OH)D (calculated free 25(OH)/total 25(OH)D) by treatment group at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.024 (0.022, 0.027)	0.024 (0.022, 0.027)	0.023 (0.021, 0.026)	0.026 (0.024, 0.029)
150 000IU	0.024 (0.022, 0.027)	0.025 (0.022, 0.028)	0.026 (0.023, 0.029)	0.024 (0.022, 0.026)
500 000IU	0.025 (0.022, 0.027)	0.024 (0.021, 0.026)	0.025 (0.023, 0.028)	0.025 (0.023, 0.028)

Values are geometric means and bars represent 95% CIs

4.17 Change in percent calculated free 25(OH)D₃ (calculated free 25(OH)D₃: total 25(OH)D₃) in response to bolus dose supplementation

4.17.1 Proportional between-group changes across time points

Change in the percent calculated free 25(OH)D₃ are shown in **figure 37** and **table 25**. There was no statistically significant interaction between treatment group and time point ($P = .135$) and no overall statistically significant difference between groups ($P = .965$).

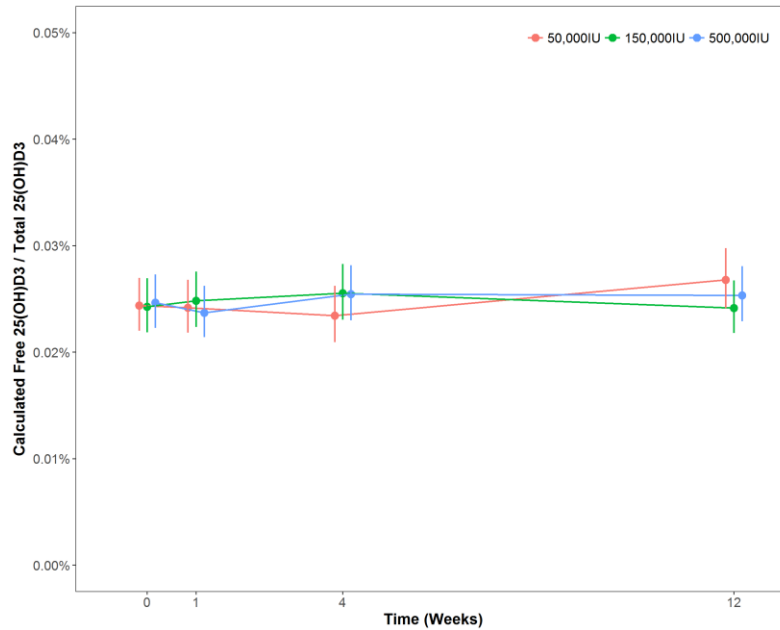
4.17.2 Proportional within-group changes across time points

In the 500 000IU treatment group the percent calculated free 25(OH)D did not change over time (ratio of difference week 1 vs. baseline: 0.96 [95% CI: 0.88, 1.06]; ratio of difference week 4 vs. baseline: 1.03 [95% CI: 0.94, 1.13]; ratio of difference week 12 vs. baseline: 1.03 [95% CI: 0.94, 1.13]).

In the 150 000IU treatment group, the percent calculated free 25(OH)D also remained similar to baseline at all weeks (ratio of difference week 1 vs. baseline: 1.02 [95% CI: 0.93, 1.13]; ratio of difference week 4 vs. baseline: 1.05 [95% CI: 0.96, 1.16]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.90, 1.10]).

The percent calculated free 25(OH)D also remained similar to baseline at all weeks in the 50 000IU group (ratio of difference week 1 vs. baseline: 0.99 [95% CI: 0.90, 1.09]; ratio of difference week 4 vs. baseline: 0.96 [95% CI: 0.87, 1.07]; ratio of difference week 12 vs. baseline: 1.10 [95% CI: 1.00, 1.21]). Absolute percent calculated free 25(OH)D values at each time point are summarised in **table 25**.

Figure 37: Percentage calculated free 25(OH)D₃ (calculated free 25(OH)D₃/total 25(OH)D₃) by treatment group



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% CIs.

Table 25: Percent calculated free 25(OH)D₃ (calculated free 25(OH)D₃/total 25(OH)D₃) by treatment group at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.024 (0.022, 0.027)	0.024 (0.022, 0.027)	0.023 (0.021, 0.026)	0.027 (0.024, 0.030)
150 000IU	0.024 (0.022, 0.027)	0.025 (0.022, 0.028)	0.026 (0.023, 0.028)	0.024 (0.022, 0.027)
500 000IU	0.025 (0.022, 0.027)	0.024 (0.021, 0.026)	0.025 (0.023, 0.028)	0.025 (0.023, 0.028)

Values are geometric means and bars represent 95% CIs.

4.18 Change in percent calculated free 1, 25(OH)₂D (calculated free 1, 25(OH)₂D: total 1, 25(OH)₂D) in response to bolus dose supplementation

4.18.1 Proportional between-group changes across time points

Change in the percent calculated free 1, 25(OH)₂D₃ are shown in **table 26**. There was no statistically significant interaction between treatment group and time point ($P = .986$). There was no overall statistically significant difference between groups ($P = .839$).

4.18.2 Proportional within-group changes across time points

In the 500 000IU treatment group the percent calculated free 1, 25(OH)₂D did not change over time (ratio of difference week 1 vs. baseline: 1.00 [95% CI: 0.81, 1.23]; ratio of difference week 4 vs. baseline: 1.07 [95% CI: 0.88, 1.33]; ratio of difference week 12 vs. baseline: 1.07 [95% CI: 0.88, 1.33]).

In the 150 000IU treatment group, the percent calculated free 1, 25(OH)₂D also remained similar to baseline at all weeks (ratio of difference week 1 vs. baseline: 1.02 [95% CI: 0.84, 1.27]; ratio of difference week 4 vs. baseline: 1.05 [95% CI: 0.86, 1.30]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.86, 1.23]).

The percent calculated free 1, 25(OH)₂D also remained similar to baseline at all weeks in the 50 000IU group (ratio of difference week 1 vs. baseline: 1.00 [95% CI: 0.80, 1.22]; ratio of difference week 4 vs. baseline: 0.91 [95% CI: 0.78, 1.18]; ratio of difference week 12 vs. baseline: 1.06 [95% CI: 0.87, 1.29]). Absolute percent calculated free 1, 25(OH)₂D values at each time point are summarised in **table 26**.

Table 26: Percent calculated free 1, 25(OH)₂D (calculated free 1, 25(OH)₂D/total 1, 25(OH)₂D) by treatment group at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.45 (0.37, 0.56)	0.45 (0.36, 0.55)	0.41 (0.035, 0.53)	0.48 (0.39, 0.58)
150 000IU	0.44 (0.36, 0.55)	0.45 (0.37, 0.56)	0.46 (0.38, 0.57)	0.44 (0.38, 0.54)
500 000IU	0.43 (0.37, 0.55)	0.43 (0.35, 0.53)	0.46 (0.38, 0.57)	0.46 (0.38, 0.57)

Values are geometric means and bars represent 95% CIs.

Discussion

4.19 Responses of total 25(OH)D and total 25(OH)D₃ to bolus dose vitamin D₃

4.19.1 Response of total 25(OH)D and total 25(OH)D₃ at week 1

As expected, there was a dose-response increase in total 25(OH)D (measured by immunoassay) and total 25(OH)D₃ (measured by LC-MS/MS) after bolus dose cholecalciferol, with rapid increases from baseline in these metabolites at 1 week post-administration. The rate of total 25(OH)D production is proportional to the amount of cholecalciferol available and this process is thought to be non-saturable (52, 225).

However, our data show that the dose-response increase in total 25(OH)D and total 25(OH)D₃ at week 1 is not linear. In the 50 000IU group, there was a 1.5-fold increase in total 25(OH)D from baseline at week 1. In the 150 000IU group, despite a three-fold higher dose, there was a 2.2-fold increase in total 25(OH)D. In the 500 000IU group, there was a 4.5-fold increase in total 25(OH)D. So, administration of a ten times higher dose, only lead to a three-fold higher increment in total 25(OH)D. The data for total 25(OH)D₃ are proportionally similar, with a 2.3-fold, 3.4-fold and 7.2-fold increases in the 50 000IU, 150 000IU and 500 000IU groups, respectively.

These findings suggest that as the dose increases there is a greater upregulation of catabolic degradation pathways for 25(OH)D. As more vitamin D₃ is hydroxylated to 25(OH)D, there is upregulation of the 24-hydroxylase enzymes (CYP24), inactivating 25(OH)D and therefore limiting the conversion of 25(OH)D to the active hormone (52, 57, 227). This ultimately limits the physiological action of 1, 25(OH)₂D (52, 57, 227).

However, Heaney et al also postulate that the non-linear response to vitamin D₃ supplementation is, in part, because the conversion of vitamin D₃ may actually be saturable in certain conditions and that the total 25(OH)D concentration in response to the input of vitamin D₃ is biphasic, and that at a higher vitamin D₃ input, 25-hydroxylation is slower (175).

4.19.2. Response of total 25(OH)D and total 25(OH)D₃ to bolus dose vitamin D₃ at weeks 4 and 12

Total 25(OH)D and total 25(OH)D₃ had peaked by week 4 in all treatment groups and remained significantly different between treatment groups at all time points. By week 12, total 25(OH)D and total 25(OH)D₃ remained higher than baseline levels in all treatment groups, but only the 500 000IU

treatment group had a significantly higher total 25(OH)D and total 25(OH)D₃ levels than the control group. The 150 000IU treatment group had total 25(OH)D and total 25(OH)D₃ levels that were comparable to those in the control group at the end of the study period, but total 25(OH)D and 25(OH)D₃ were significantly lower in the 50 000IU treatment group than control levels.

This relatively slow decline in total 25(OH)D and 25(OH)D₃ from peak levels at weeks 1 and 4 to week 12 in all treatment groups can be explained by slow release of vitamin D₃ from storage depots, such as fat (175). When there is excess vitamin D₃, such as after a large single bolus dose, not all is converted and used metabolically, but some is distributed into fat and other non-adipose tissues (143, 175).

Interestingly, in this study we do see a greater fall in total 25(OH)D concentration at week 12 from previous time points in the 500 000IU group compared to the lower doses and this may be due to a greater upregulation of degradation pathways for 25(OH)D in the higher dose group (see chapter 5).

The best current model to explain this release of vitamin D₃ into circulation from adipose tissue stores is a passive diffusion dilutional model proposed by Drincic et al, in which equilibrium is reached between vitamin D₃ dissolved in adipose tissue and other stores and vitamin D₃ bound to VDBP in the serum (143). In this model it is postulated that equilibrium is reached between the VDBP-bound vitamin D₃ and the vitamin D₃ in adipose tissues, with respective relative affinities for vitamin D₃ of approximately 1:12 (143, 315). In this volumetric dilution model, hepatic hydroxylation of vitamin D₃ acts as a competitive pathway, drawing the vitamin D₃ from adipose tissue when a fraction of VDBP-bound D₃ is taken up by the liver (143). This is supported by the relatively lower total 25(OH)D levels in obese individuals (20) and the relatively lower increment in total 25(OH)D in obese people compared to normal weight individuals after vitamin D supplementation (174). The persistence of hypercalcemia for several months after known vitamin D intoxication may also be due to a sustained release of vitamin D₃ from body stores (175).

The total 25(OH)D & 25(OH)D₃ only remained above the sufficiency threshold (25(OH)D > 50nmol/l) in the 500 000IU (geometric mean: 91.0nmol/l [95% CI: 80.4, 103.0] and 77.4nmol/l [95% CI: 69.1, 86.7], respectively) and 150 000IU (geometric mean: 58.1nmol/l [95% CI: 51.1, 66.1] & 51.8nmol/l [95% CI: 46.2, 58.0], respectively) treatment groups by 12-weeks. Total 25(OH)D in the 50 000IU treatment group only just reached the sufficiency threshold at week-4 (53.1nmol/l, [95%CI: 46.7, 60.4] and total 25(OH)D₃ did not reach the sufficiency threshold at any time point in this treatment group. This indicates that a minimum bolus of 150 000IU vitamin D₃ is required to replete vitamin D deficient patients and then maintain their total 25(OH)D levels above 50nmol/l during a 3-month period in the depths of winter where cutaneous synthesis is limited.

The control group levels of total 25(OH)D and total 25(OH)D₃ did not fall significantly from baseline levels despite the study being carried out over the winter period where no significant cutaneous synthesis is possible in the UK, and lower than recommended dietary vitamin D intake. This may be due to underestimation of vitamin D intake, or maintenance of serum levels through release from tissue storage.

4.19.3 Total 25(OH)D and 25(OH)D₃ profile in the 500 000IU treatment group vs. other literature

In the 500 000IU treatment group there were sharp increases from baseline at week 1 of total 25(OH)D and total 25(OH)D₃ of +111.5nmol/l and +132.4nmol/l as measured by immunoassay and LC-MS/MS, respectively. Changes in total 25(OH)D from baseline remained similar at 4- weeks post-administration at +100.6nmol/l. However, total 25(OH)D₃ measured by LC-MS/MS had fallen at week 4 (+97.8nmol/l vs. baseline). Total 25(OH)D had declined by week 12 and total 25(OH)D₃ had declined further, but both measures remained above baseline levels.

Despite the heterogeneity between studies (including differences in population sub-groups, total 25(OH)D measurement method & study time points), the changes in total 25(OH)D in this study are in line with other bolus dosing studies in healthy people (8, 213).

Results from studies that have administered a slightly higher bolus doses of 600 000IU also show comparable increments in total 25(OH)D to the current study. Cipriani et al reported an increase in total 25(OH)D of approximately +153nmol/l and +117nmol/l at 3 days and 30 days post- administration, respectively, in vitamin D insufficient younger adults (173). Rossini et al, reported a slightly smaller increase in total 25(OH)D of approximately +101nmol/l and +75nmol/l at 7 days and 30 days, respectively, but this was in elderly vitamin D sufficient participants (219).

4.19.4 Total 25(OH)D and 25(OH)D₃ profile in the 150 000IU treatment group vs. other literature

In the 150 000IU treatment group there were increases from baseline at week 1 of total 25(OH)D and total 25(OH)D₃ of +38.6nmol/l and +49.1nmol/l, respectively. Changes in total 25(OH)D and total 25(OH)D₃ from baseline remained similar at 4- weeks post-administration at +42.7nmol/l and 48.7nmol/l, respectively. Total 25(OH)D and total 25(OH)D₃ fell by week 12 but remained above baseline levels (+26nmol/l and +31.3nmol/l, respectively)

The changes to total 25(OH)D in the 150 000IU treatment group are also in line with changes reported in other studies, despite heterogeneity in design. Glendenning et al reported an absolute basal increase in total 25(OH)D of +9nmol/l 3-months after administration of a 150 000IU bolus in vitamin D deficient

community dwelling older women (aged 76.9 +/- 4.0) (237). The smaller increment from baseline in total 25(OH)D in this study may be explained by the difference in baseline vitamin D status and or/ difference in total 25(OH)D measurement methods used between studies.

4.19.5 Total 25(OH)D and 25(OH)D₃ profile in the 150 000IU treatment group vs. other literature

In the 50 000IU treatment group there were smaller increases from baseline at week 1 of total 25(OH)D and total 25(OH)D₃ of +17.7nmol/l and +26.7nmol/l, respectively. Changes in total 25(OH)D and total 25(OH)D₃ from baseline remained similar at 4- weeks post-administration at +22.7nmol/l and +26.3nmol/l, respectively. Total 25(OH)D and total 25(OH)D₃ fell by week 12 but remained above baseline levels (+13.9nmol/l and +13.2nmol/l, respectively). These changes are similar to those reported in other studies that have administered a similar dose (213).

4.20 Response of total 1, 25(OH)₂D to bolus dose vitamin D₃

4.20.1 Response of total 1, 25(OH)₂D to bolus dose vitamin D₃ at week 1

As expected, there was also a dose-response increase in total 1, 25(OH)₂D after bolus dose cholecalciferol at 1 week post-administration, with a particularly large increase in the higher dose group. This is to be expected because as total 25(OH)D increases in the serum, the CYP27B1 (1- α -hydroxylase) enzymes in the kidney produces increasing amounts of 1, 25(OH)₂D per unit of enzyme protein (226).

These findings are also in line with other studies that have measured 1, 25(OH)₂D after a single 600 000IU bolus of vitamin D₃ that have also demonstrated rapid increases in the active metabolite as early as 3- days post-supplementation (172, 214, 313). The basal increase in total 1, 25(OH)₂D in these studies of around 50pg/ml just 3-days after administration (214, 313) is similar to the 36pg/ml increase that we see in this study with a slightly lower 500 000IU bolus at 1-week post-administration.

There were also smaller increases in total 1, 25(OH)₂D at 1-week after administration from baseline levels in the 150 000IU and 50 000IU treatment groups. However, other studies that have used bolus doses of vitamin D₃ up to 200 000IU have failed to find any changes in total 1, 25(OH)₂D at 1-week post-administration (245, 311), although these studies were carried out in participants who were already vitamin D sufficient.

As with total 25(OH)D, the dose-response for 1, 25(OH)₂D at week 1 was not linear, and the proportional increases in total 1, 25(OH)₂D in each treatment group (500 000IU: 2.3-fold; 150 000IU: 1.8-fold; 50 000IU 1.3-fold) were less than total 25(OH)D. This is expected because of the tight regulation of the 1- α -

hydroxylase and 24-hydroxylase enzymes by PTH and FGF-23 (58, 59, 225). Although, we did not see a dose-response change in PTH in this study, there was an increase in FGF-23 in two of the treatment groups (see chapter 5).

4.20.2 Response of total 1, 25(OH)₂D to bolus dose vitamin D₃ at weeks 4 and 12

In the 500 000IU group, there was a sharp fall in total 1, 25(OH)₂D at week 4 from week 1 and remained at similar levels at week 12. However, total 1, 25(OH)₂D remained above baseline levels at the end of the study. In the 150 000IU and 50 000IU treatment groups total 1, 25(OH)₂D was similar to week 1 at week 4 and levels had fallen slightly by week 12 but remained higher than baseline in each group.

The increase in total 1, 25(OH)₂D at week 1 before falling sharply at week-4 in the highest dose group also supports a shift to the induction of the degradation pathways for the 25(OH)D and 1, 25(OH)₂D metabolites. There is a direct negative feedback on 1, 25(OH)₂D production by 1,25(OH)₂D through the downregulation of gene expression for CYP27B1 (52). Therefore, when circulating levels of 1, 25(OH)₂D increase, then renal production decreases (2); this would help to explain the sharp fall that we see in the highest dose group at week 4 and the plateau in the lower dose groups.

These findings are also in line with other bolus dosing studies that have reported a decrease in total 1, 25(OH)₂D after an initial sharp increase from baseline levels (214, 313). The approximate -20-23pg/ml fall in total 1, 25(OH)₂D at 30-days post-administration of a 600 000IU bolus from 3-days post-administration is very similar to the fall in total 1, 25(OH)₂D of -18.3pg/ml that we see from 1-week to 4-weeks in the 500 000IU treatment group in this study. Also, in line with findings in this study, total 1, 25(OH)₂D was reported to still be above baseline 60-days post-administration in the three studies by the Cipriani group (173, 214, 313).

Total 1, 25(OH)₂D in the 500 000IU treatment group remained higher than control group levels at the end of the study. The active metabolite was lower than control levels at the end of the study in the 50 000IU group, but no difference was found between control and the 150 000IU treatment group. This is in line with the pattern established for the total 25(OH)D.

Overall these findings do not support the hypothesis that any adverse effects of a single large bolus of vitamin D may be caused by a decrease in circulating total 1, 25(OH)₂D (an ultimately tissue levels of 1, 25(OH)₂D) triggered by a protective over compensation in the upregulation of the CYP24 enzyme responsible for the catabolism of 1, 25(OH)₂D (222). Also total 1, 25(OH)₂D remained in the normal range throughout the entire study period in all treatment groups and at all time points, suggesting that

the total $1, 25(\text{OH})_2\text{D}$ remains under tight hormonal regulation even at single oral doses of up to 500 000IU (and up to 600 000IU reported elsewhere). Therefore, the total $1, 25(\text{OH})_2\text{D}$ metabolite does not appear to be the metabolite that is likely to trigger any adverse events after a 500 000IU D_3 bolus, such as those reported by Sanders et al (8).

4.21 Response of free $25(\text{OH})\text{D}$ to bolus dose vitamin D_3

4.21.1 Response of free $25(\text{OH})\text{D}$ to bolus dose vitamin D_3 at week 1

Measured free $25(\text{OH})\text{D}$ rose in a dose dependent manner and the response profile for directly measured free $25(\text{OH})\text{D}$ was similar to that of total $25(\text{OH})\text{D}$ and total $25(\text{OH})\text{D}_3$. Both measures of calculated free $25(\text{OH})\text{D}$ also showed a similar dose-response profile. This is in line with expectations as there is a strong correlation between total $25(\text{OH})\text{D}$ and free $25(\text{OH})\text{D}$ in healthy participants whether measured directly or calculated (156, 165, 314). Comparison of the absolute values between the measured and calculated free $25(\text{OH})\text{D}$ at each time point and in each treatment group is remarkably consistent, although calculated values tended to be slightly higher than the directly measured values. This is also consistent with other literature and it has been postulated that this may be due to uncertainty in the association constants that are used to calculate free $25(\text{OH})\text{D}$ (165).

The dose response of free $25(\text{OH})\text{D}$ is not linear, just as the total $25(\text{OH})\text{D}$ and $25(\text{OH})\text{D}_3$ response. For measured free $25(\text{OH})\text{D}$, In the 50 000IU treatment group we observed a 1.7-fold increase from baseline at week 1. In the 150 000IU group, we only observe a slightly higher 2.1-fold increase in total $25(\text{OH})\text{D}$ and in the 500 000IU group there was a 7.1-fold increase in directly measured free $25(\text{OH})\text{D}$. The proportional increases in free $25(\text{OH})\text{D}$ derived by calculated methods also demonstrate similar fold increases. Calculated free $25(\text{OH})\text{D}$ increased 1.6-fold, 2.3-fold and 4.3-fold, in the 50 000IU, 150 000IU and 500 000IU groups, respectively. Calculated free $25(\text{OH})\text{D}_3$ increased 2.3-fold, 3.5-fold and 6.9-fold, in the 50 000IU, 150 000IU and 500 000IU groups, respectively. These proportional increases are completely with the proportional increases in total $25(\text{OH})\text{D}$ (50 000IU: 1.6-fold; 150 000IU: 2.2-fold; 500 000IU 4.5-fold) and total $25(\text{OH})\text{D}_3$ (50 000IU: 2.3-fold; 150 000IU: 3.4-fold; 500 000IU 7.2-fold) at 1-week post-administration. Overall, this data would indicate that there is no disproportional rise in free $25(\text{OH})\text{D}$ after a single large bolus of vitamin D_3 in this study and that the proportional increases in free $25(\text{OH})\text{D}$ are comparable to total $25(\text{OH})\text{D}$.

Only one other study to date has reported the response of free $25(\text{OH})\text{D}$ after a bolus dose of vitamin D_3 and this was done using the calculated method (245). After a 100 000IU bolus of vitamin D_3 , calculated free increased from 15.5pmol/l (range: 8.0-69.8) to 27.8pmol/l (range: 15.5-105.5) in vitamin D

sufficient healthy controls (n= 29) and increased from 9.5pmol/l (range: 3.5-40.5) to 15.5pmol/l (range: 5.8-63.3) in burns patients (n = 20) 1-week after administration (245). The 1.8-fold and 1.6-fold increases in healthy controls and burns patients, respectively, are slightly lower than the proportional increases that were found in directly measured and calculated 25(OH)D in the 150 000IU treatment group at week 1 in this study.

The percentage free 25(OH)D (derived from the ratios of calculated free 25(OH)D to total 25(OH)D and calculated free 25(OH)D₃ to total 25(OH)D₃ at week-1 post administration (and at all other study time points) were not different between treatment groups at any time point and do not change across the study period. The percentage free that was derived from the ratio of measured free 25(OH)D to total 25(OH)D show a slight increase from baseline at week 1 in the 500 000IU treatment group that was significantly different compared to the other treatment groups. However, the percent free remained in the normal range and comparable with percentages reported in healthy adults (0.02-0.09%) (156, 165). The percent free reported by Rousseau et al was also unchanged from baseline at 1-week post-administration at 0.03% and 0.04% in the healthy controls and burns patients, respectively (245). These percentage free totals in the healthy controls are comparable to those reported in this study.

4.21.2 Response of free 25(OH)D to bolus dose vitamin D₃ at weeks 4 and 12

In the 500 000IU group, all measures of free 25(OH)D remained similar to week 1 at week 4, before steadily declining by week 12. For all measures of free 25(OH)D in the highest treatment group, the concentration of free 25(OH)D remained above baseline levels at week 12. In the 150 000IU group, all measures of free 25(OH)D held at week 1 levels for the remainder of the study period, except for a small fall at week 12 from week 4 in calculated free 25(OH)D₃. For all measures of free 25(OH)D in the 150 000IU treatment group, the concentration of free 25(OH)D remained above baseline levels at the end of the study. In the 50 000IU group, all measures of free 25(OH)D held at week 1 levels for the remainder of the study period and the concentration of free 25(OH)D remained above baseline levels at the end of the study.

All measures of free 25(OH)D remained significantly higher in the 500 000IU group and lower in the 50 000IU group compared to control group levels at week 12. All measures of free 25(OH)D in the 150 000IU group were not different to control levels at week 12.

Despite these differences, all measures of free 25(OH)D remained in the normal range for absolute values of free 25(OH)D and percent free 25(OH)D also did not differ between treatment groups and did

not change within any of the treatment groups across time points. This further reinforces that a single large D₃ bolus dose of up to 500 000IU did not cause any abnormal increases in free 25(OH)D.

4.22 Response of free 1, 25(OH)₂D to bolus dose vitamin D₃

4.22.1 Response of free 1, 25(OH)₂D to bolus dose vitamin D₃

No other study to date has reported calculated free 1, 25(OH)₂D in response to a single large bolus dose of vitamin D₃. In this study, calculated free 1, 25(OH)₂D increased in a dose dependent manner and the response profile for calculated free 1, 25(OH)₂D was similar to that of the 25(OH)D metabolites, with sharp increases at week 1 from baseline, particularly in the 500 000IU group. There was a fall in free 1, 25(OH)₂D by week 4 in the 500 000IU group and levels remained similar at week 12. Levels remained similar to the respective week 1 concentrations in the 50 000IU and 150 000IU groups at weeks 4 and 12.

As demonstrated for the other vitamin D metabolites, the dose response of free 1, 25(OH)₂D is not a linear one. In the 50 000IU treatment group we observed a 1.3-fold increase from baseline at week 1. In the 150 000IU group, we only observe a slightly higher 1.8-fold increase and in the 500 000IU group there was a 2.2-fold increase in free 1, 25(OH)₂D. These are remarkable in similar to the 1.3-fold, 1.8-fold and 2.3-fold increases in total 1, 25(OH)₂D from baseline at week-1.

It has been proposed that in vitamin D toxicity the concentration of vitamin D metabolites (such as D₃ and 25(OH)D) exceed the capacity of the VDBP and that this causes the release of free 1, 25(OH)₂D due to its relatively lower binding affinities for VDBP compared to these other vitamin D metabolites) (47). Thus, the ability of VDBP to restrict cellular entry of 1, 25(OH)₂D becomes impaired and unwanted biological effects may occur due to the high affinity of 1, 25(OH)₂D with the VDR. In this scenario of toxicity, free 1, 25(OH)₂D may cause increased gene activation and transcription (47).

However, our data would indicate that there is no disproportional rise in free 1,25(OH)₂D after a single large bolus of vitamin D₃ at the doses used in this study and does not support the hypothesis that adverse events after a single large bolus of the sizes used in this study are caused by excess free 1, 25(OH)₂D. The findings from this study do not dispute elevated free 1,25(OH)₂D concentrations may be implicated in vitamin D toxicity in certain conditions when total 25(OH)D concentrations reach much higher levels than those reached in this study, such as those reported in case studies of toxicity (> approximately 535nmol/l)(225, 228, 316).

The percent free 1, 25(OH)₂D did not change from baseline in any treatment group and did not differ between treatment groups at any time point. The percent free 1, 25(OH)₂D in all treatment groups and at all time points was also in line with the 0.4% reported by other authors in healthy participants (156). This also reinforces the conclusion that free 1, 25(OH)₂D does not lead to adverse events found in the sanders et al study (8).

4.23 Chapter considerations - strengths and limitations

This is the first study to try and elucidate the mechanism of why people might fall and fracture more after a single large bolus dose as reported by Sanders et al (8).

Total 25(OH)D has been measured using more than one method, including measurement by the gold standard LC-MS/MS method. This is only the second study to report total 25(OH)D₃ measured by LC-MS/MS after a bolus dose of vitamin D₃ at ranges up to 500 000IU (only one previous study reported total 25(OH)D₃ measured by LC-MS/MS, but this was after a bolus of only 100 000IU). The consistency of the response profiles of the different vitamin D metabolites achieved across the different measurement methods for each metabolite is reassuring and gives confidence to conclusions made from the data.

There is a paucity of data on total 1,25(OH)₂D and free metabolites after bolus dosing in the existing literature. A variety of methods have been used to measure and calculate free 25(OH)D in this study to give a comprehensive assessment of this metabolite. This is the first study to report serial measurements of directly measured and calculated free 25(OH)D and calculated free 1, 25(OH)₂D in response to a single large bolus dose.

We did not measure DBP genotype and so it is possible that there are variations in the VDBP genotype in our participants that may give rise to different binding affinities affecting calculated free metabolite results. However, we used a Caucasian population only and measured VDBP with the superior polyclonal antibody assay, which limits variation in calculated free 25(OH)D. We also found that the calculated methods using the VDBP concentrations measured by the polyclonal assay were comparable to the measured free 25(OH)D. This is in line with reports from other studies (165).

Based on data from other bolus dosing studies that had previously been carried out and to reduce participant burden an initial post-administration time point of 5-7 days was determined as this was estimated to be the point in which we would see peak concentrations of total 25(OH)D. However, large increases have been reported as early as 3 days after a 600 000IU dose (173) and therefore it is plausible that we may not have captured the absolute peak total 25(OH)D and the peak concentrations of other metabolites.

The levels of total 25(OH)D achieved in this study after the largest bolus would appear to not be high enough to saturate VDBP in circulation, as animal studies and human studies of vitamin D intoxication have reported total 25(OH) levels of ~500nmol/l and above are required to achieve a displacement of free metabolites from the VDBP and to cause hypercalcemia (47, 225, 228, 316, 317) Despite this, the increases in total 25(OH)D that we have reported are certainly in line with those reported in other studies that had demonstrated adverse effects of large doses of vitamin D. In the 500 000IU group the total 25(OH)D achieved is similar to thresholds that have been associated with adverse events in other studies (10, 223).

Chapter conclusions

The study aimed to investigate whether there is a disproportionate rise in free 25(OH)D and free 1, 25(OH)₂D after a single large bolus dose of vitamin D₃ to determine if these metabolites might play a role in explaining the increase in falls and fracture in the three-month period after a 500 000IU bolus dose.

In response to three different single bolus doses of vitamin D₃ (50 000IU, 150 000IU and 500 000IU), in vitamin D deficient, but healthy older participants, total 25(OH)D increased in a dose dependent manner at 1-week post administration. Total 25(OH)D remained at similar levels up to 4 weeks after administration, before falling at week 12. The other vitamin D metabolites, including total 1, 25(OH)₂D, measured and calculated free 25(OH)D and calculated free 1, 25(OH)₂D had similar dose-response profiles to total 25(OH)D (and 25(OH)D₃).

The dose-response of all vitamin D metabolites was not linear and is likely due to the upregulation of catabolic pathways in response to increases in the serum concentration of the 25(OH)D and 1, 25(OH)₂D metabolites.

The proportional increases in free 25(OH)₂D (either calculated or directly measured) are in line with the proportional increases in total 25(OH)D (and 25(OH)D₃) across all treatment groups and time points. The percent free 25(OH)D also remained within previously described reference ranges in all treatment groups and at all time points. The proportional changes in calculated free 1, 25(OH)₂D were also similar to the proportional changes in total 1, 25(OH)₂D across all treatment groups and at all study time points. There was also little change in the percent free 1, 25(OH)₂D between treatment groups and across study time points.

Taken together, the data presented indicates that there is little evidence to support the hypothesis of a disproportionate rise in free 25(OH)D or free 1, 25(OH)₂D after a single large bolus dose in this vitamin D deficient, but otherwise healthy, older population. It is therefore, unlikely that the adverse events reported by Sanders et al (8) after a 500 000IU oral bolus, and in other studies that have reported similar adverse events to large doses of vitamin D, are caused by excess or disproportionate levels of free vitamin D metabolites.

We must take some caution in making this conclusion due to the slightly younger and likely less frail population used in this study compared to other studies that have reported adverse events after vitamin D bolus dosing. This study also does not exclude a role for free metabolites, particularly free 1, 25(OH)₂D, in vitamin D toxicity when serum total 25(OH)D concentrations are in excess of the binding capacity threshold of around 500nmol/l and above. However, in this bolus dosing study (and other studies reporting adverse events after bolus dosing) total 25(OH)D levels do not reach such high levels and therefore we see no disproportionate rise in the free metabolites of 25(OH)D and 1, 25(OH)₂D and no change in the percent free of these metabolites.

Therefore, the cause of the adverse events after a large bolus dose of vitamin D that has been demonstrated in some studies remains unknown.

Chapter 5:

Effects of bolus dose cholecalciferol on calcium metabolism, bone turnover markers and muscle and cardiovascular parameters

Chapter overview

This chapter will investigate changes in calcium metabolism, bone turnover markers, and cardiovascular parameters (research questions 3-6) after administration of oral bolus dose vitamin D₃ in vitamin D deficient postmenopausal women. This will lead to a greater understanding out how these outcomes respond to bolus dose vitamin D₃. It will also allow testing of the hypothesis that a single large bolus doses of vitamin D₃ may lead an increase in falls (and fracture) because of adverse effects such as elevated serum total and ionised calcium, poorer physical function and postural hypotension.

5.0 Research questions, aims and hypotheses

5.1 Research Questions

1. How do different bolus doses of vitamin D₃ affect carrier proteins and measures of calcium metabolism in vitamin D deficient postmenopausal women?
2. How do different bolus doses of vitamin D₃ affect bone turnover markers in vitamin D deficient postmenopausal women?
3. How do different bolus doses of vitamin D₃ affect measures of muscle function and muscle strength in vitamin D deficient postmenopausal women?
4. How do different bolus doses of vitamin D₃ affect cardiovascular parameters in vitamin D deficient postmenopausal women?

5.2 Aims

1. To determine the response of albumin and vitamin D binding protein to different bolus doses of vitamin D₃ in vitamin D deficient postmenopausal women.
2. To determine the response of PTH, sCa, iCa, sPhos, and 24-hour urCa:urCr to different bolus doses of vitamin D₃ in vitamin D deficient postmenopausal women.
3. To determine the response of PINP, OC and CTX to different bolus doses of vitamin D₃ in vitamin D deficient postmenopausal women.
4. To determine effect of different bolus doses of vitamin D₃ on SPPB scores and grip strength in vitamin D deficient postmenopausal women.
5. To determine the effect of different bolus doses of cholecalciferol on systolic and diastolic blood pressure, laying and standing pulse rate and renin and aldosterone levels in vitamin D deficient postmenopausal women.

5.3 Hypotheses

1. There will be a dose-response rise in albumin and vitamin D binding protein after bolus dose supplementation of vitamin D₃ 1-week after administration
2. There will be a dose-response decrease in PTH in response to bolus dose supplementation
3. There will be an increase in serum calcium and serum ionised calcium in the largest bolus dose group at 1-week after administration
4. There will be a dose-response increase in 24-hour urCa:urCr in response to bolus dose supplementation
5. There will be a dose-response increase in PINP, OC and CTX in response to bolus dose supplementation
6. There will be an improvement in SPPB and grip strength scores in the lower dose groups, but the higher dose group will have a negative effect on SPPB and grip strength scores
7. There will be a dose-response decrease in systolic and diastolic blood pressure and ARR in response to bolus dose supplementation

5.1 PTH response to bolus dose vitamin D₃

A limited number of studies have investigated the effect of a single large bolus of vitamin D₃ on PTH. A summary of these studies is shown in **table 27**.

5.1.2 Single large bolus dose $\geq 500\ 000\text{IU}$

PTH has been reported to significantly decrease from baseline values as early as 3-days post administration of a 600 000IU Bolus of Vitamin D₃, with levels still significantly below baseline at 90-days after administration (214, 219, 313). Tellioglu et al also report significant decreases in PTH from baseline at 6- and 12-weeks after a 600 000IU D₃ bolus (212). In contrast, Cipriani et al only report significant decreases in PTH 30 days, but not at 60-, 90- or 120-days after administration of a 600 000IU D₃ bolus in healthy older participants (173). Bacon et al also report significant reductions in PTH at 1-month after administration after a 500 000IU dose, but only in those who has a baseline 25(OH) <20nmol/l (213).

5.1.2 Single large bolus dose $\geq 200\ 000\text{IU}$ $\geq 300\ 000\text{IU}$

A sharp decrease in PTH was found just 3-days after administration of a 300 000IU D₃ bolus and PTH remained significantly lower than baseline at 60-days follow-up in elderly nursing home residents (172). PTH was also lower than baseline at 1-month after a 300 000IU D₃ bolus in elderly patients with secondary hyperparathyroidism and remained lower than baseline at 9-months (201). PTH has also seems to remain significantly lower than baseline at 3- and 6-months after administration of a 300 000IU D₃ bolus in vitamin D deficient older (216, 217) and insufficient younger adults (215).

After a slightly lower D₃ bolus of 200 000IU in healthy vitamin D sufficient older participants, PTH was significantly lower than baseline 1-week (311) and 1-month (312) after administration. However, PTH had returned to baseline levels by 3-months (311). In contrast, there was no significant between group differences in PTH at any time point (5-, 90- and 365-days) after a 250 000IU D₃ bolus compared to placebo in vitamin D insufficient young healthy adults (218).

5.1.3 Single large bolus dose <200 000IU

A significant decrease in PTH has been reported just 3-days after administration of a 100 000IU D₃ bolus in vitamin D sufficient community dwelling older adults (219). However, PTH had returned to baseline levels by 7-days after administration (219). Similar findings have been shown at 1-week after a 100 000IU D₃ bolus in healthy younger adults (245). PTH also did not change across study follow-up time points (1-, 2- and 3-months post administration) in vitamin D sufficient older community dwelling participants after a ~100 000IU D₃ bolus (200). PTH was also similar to baseline values at the end of the 60-day study period after a 100 000IU in healthy older participants (286).

Table 27: A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH		PTH Response		
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
Single large bolus dose ≥ 600 000IU										
Cipriani et al., (2010) (214)	48	Healthy young adults aged: 36 +/- 8	M/F	600 000IU D ₃	Single oral bolus dose	40 +/- 16	53.0 +/- 20.0	3-days	193 +/- 76	38.6 +/- 17.2 ^a
								15-days	191 +/- 70	40.6 +/- 15.8 ^a
								30-days	156 +/- 65	43.4 +/- 14 ^a
								60-days	107 +/- 22	42.8 +/- 19.1 ^a
								90-days	80 +/- 32	37.7 +/- 15.1 ^a
Cipriani et al., (2013) (173)	6	Healthy participants aged: 63.9 +/- 7.1	M/F	600 000IU D ₃	Single oral dose	52 +/- 16	38.7 +/- 8.0	30-days	+31 +/- 11 basal increase	Data not shown but report a significant decrease in PTH only at 30-days
								60-days	79 +/- 12	
								90-days	70 +/- 10	
								120-days	Approximately baseline level	
Cipriani et al., (2013) (313)	18	Healthy but previously undergone surgery for localised melanoma aged: 32.1 +/- 4.7	F	600 000IU D ₃	Single oral bolus dose	45 +/- 19	57.6 +/- 22.7	3-days	188 +/- 42	39.4 +/- 16.4 ^a
								15-days	184 +/- 34	35.6 +/- 14.0 ^a
								30-days	162 +/- 31	38.5 +/- 13.4 ^a
								60-days	107 +/- 23	42.2 +/- 18.8 ^a
								90-days	80 +/- 33	37.8 +/- 15.9 ^a

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range

Table 27 (cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
Single large bolus dose ≥ 600 000IU										
Tellioglu et al., (2012) (212)	32	75.3 +/- 7.5 Ambulatory nursing home participants	M/F	600 000IU D ₃	Single oral dose	37.2 +/- 13.8	52.0 +/- 22.5	6-weeks 12-weeks	119 +/- 32 107 +/- 34	PTH decreased significantly from baseline at weeks 6 and 12 (P<.0001)
Rossini et al., (2012) (219)	37	Elderly community dwelling aged: 75 +/- 3	M/F	(1) 600 000IU D ₃ (n= 12) (2) 300 000IU D ₃ (n= 12) (3) 100 000IU D ₃ (n= 13) (4) Control (n= 24)	Single oral bolus doses	(1) 54 +/- 14 (2) 65 +/- 17 (3) 66 +/- 19 (4) 55 +/- 18	(1) 35.0 +/- 8.7 (2) 30.9 +/- 7.6 (3) 31.6 +/- 7.5 (4) 33.2 +/- 7.9	1-day 3-days 7-days 14-days 30-days 60-days 90-days	(1) 117 +/- 19; (2) 84 +/- 21; (3) 82 +/- 24 (1) 168 +/- 43; (2) 95 +/- 24; (3) 84 +/- 20 (1) 156 +/- 31; (2) 96 +/- 23; (3) 86 +/- 21 (1) 152 +/- 33; (2) 95 +/- 22; (3) 83 +/- 20 (1) 129 +/- 30; (2) 86 +/- 21; (3) 84 +/- 21; (4) 55 +/- 16 (1) 108 +/- 26; (2) 77 +/- 16; (3) 85 +/- 20; (4) 61 +/- 17 (1) 88 +/- 14; (2) 72 +/- 15; (3) 79 +/- 20; (4) 58 +/- 15	(1) 32.0 +/- 9.5; (2) 27.6 +/- 7.9; (3) 30.6 +/- 6.8 (1) 25.5 +/- 7.4 ^a ; (2) 25.4 +/- 6.8 ^a ; (3) 26.0 +/- 5.6 ^a ; (4) 34.6 +/- 6.5 (1) 23.4 +/- 6.4 ^a ; (2) 27.2 +/- 6.6 ^a ; (3) 28.7 +/- 8.0; (4) 32.9 +/- 7.8 (1) 25.8 +/- 7.8 ^a ; (2) 28.1 +/- 10.0; (3) 29.8 +/- 8.2; (4) 33.8 +/- 7.6 (1) 27.0 +/- 9.8 ^a ; (2) 25.7 +/- 7.9 ^a ; (3) 28.2 +/- 6.2; (4) 31.7 +/- 7.3 (1) 29.3 +/- 6.0 ^a ; (2) 26.3 +/- 6.1 ^a ; (3) 29.0 +/- 7.2; (4) 33.8 +/- 6.2 (1) 28.3 +/- 6.1 ^a ; (2) 27.2 +/- 8.8; (3) 30.7 +/- 7.2; (4) 34.7 +/- 7.7

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range

Table 27 (cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
Single large bolus dose = 500 000IU										
(Bacon et al., 2009) (213)	19	82+/- 7 recently hospitalized		500 000IU D ₃	Single oral bolus	58 +/- 25	48.1 +/- 22.6	1-, 2-, 3-, 4-, 5-, 6-, 7-, 8 & 9-months	+58 basal increase at 1 month with gradual decline until plateau of 69 +/-6 at 9 months	Decreased by 9.4 at 1 month (P = .0075) In pooled analysis, when 25(OH)D <20nmol/l there were substantial reductions in PTH, but at higher 25(OH)D levels supplementation did not affect PTH

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range

Table 27 (cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
Single large bolus dose >200 000IU > 300 000IU										
Leventis & Kiely (2009) (215)	19	Vitamin D insufficient aged: (2) 43 (23-72)*	M/F	300 000IU D ₃	Single oral dose	27 (5-40)*	52.6 (24.5-83.9)	6-weeks 12-weeks 24-weeks	135 (65-214) 82 (47-119) 43 (23-78)	Not measured 40.6 (20.0-54.7) ^a 41.2 (26.4-55.6) ^a
Giusti et al., (2010) (217)	30	Community dwelling older adults aged: 74 +/- 6	F	300 000IU D ₃	3-monthly oral bolus dose	23 +/- 12	99.9 +/- 51.9	3-months 6-months	52 +/- 32 80 +/- 30	78.0 +/- 34.7 ^a 72.9 +/- 38.1 ^a
Cavalcante et al., (2015) (312)	20	Elderly aged: 68 +/- 6 years	F	(1) 200 000IU D ₃ (2) Placebo	Single oral dose	(1) 63 +/- 7 (2) 65 +/- 9	(1) 46.3 +/- 13.2 (2) 34.19 +/- 10.0	4-weeks	(1) 79 +/- 15 (2) 61 +/- 10	(1) 35.5 +/- 11.0 ^a (2) 38.2 +/- 14.9
Von Restorff et al., (2009) (216)	33	Elderly with 25(OH)D <25nmol/l aged: 81 +/- 6	M/F	300 000IU D ₃	Single oral dose	15 +/- 6	75.9 +/- 86.9	3-months 6-months	81 +/- 30 69 +/- 18	58.9 +/- 39.8 ^a 60.0 +/- 42.7 ^a

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range

Table 27(cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (ng/ml) at follow-up
Single large bolus dose >200 000IU > 300 000IU										
Romagnoli et al., (2008) (172)	8	Elderly nursing home residents aged: 79 +/- 8	F	300 000IU D ₃	Single oral bolus	33 +/-25	43.8 +/-24.5	3-days 7-days 30-days 60-days	Sharp increase after 3-days and at 60 days mean levels still significantly higher than baseline (P<.01)	Sharp decrease observed at 3 days that remained significantly lower (P<.01) than baseline at 60-days (-22.8 +/- 16)
Premaor et al., (2008) (201)	14	Low socioeconomic elderly with secondary hyperparathyroidism aged: 81 +/-9	M/F	300 000IU D ₃	single oral bolus	31 +/- 17	70.6 +/- 25.0	1-month 2-months 3-months 6-months 9-months	~82 ~70 ~65 ~25 ~30	~50 ~50 ~60 ~60 ~55
Valimaki et al., (2016) (311)	40	75 +/- 2.9	F	(1) 100 000IU D ₃ (n = 20) or; (2) 200 000IU D ₃ (n = 20) (3) Placebo (n = 20)	Every three months for 1-year	(1) 60.0 (31-99)* (2) 63.5 (38-87)* (3) 61.5 (15-101)*	(1) 53.8 (17.9-151-.8)* (2) 50.0 (22.6-109.4)* (3) 50.0 (13.2-126.4)*	1-week 3-months	(1) 73 (59-119) (2) 74.5 (58-114) (3) 60 (18-102) (1) 67 (51-106) (2) 71(49-92) (3) 64 (34-92)	(1) 48.1 (25.5-313.1) (2) 34.9 (2.8-74.5) ^a (3) 43.4 (2.8-124.5) (1) 50.9 (25.5-242.4) (2) 44.3 (2.8-87.7) (3) 44.3 (2.8-134.8)

Table 27(cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
Single large bolus dose >200 000IU > 300 000IU										
Kearns et al., (2015) (218)	14	Young, healthy adults aged: (1) 28.2 +/-6.7 (2) 26.5 +/- 5.2	M/F	(1) 250 000IU D ₃ (n = 14) (2) Placebo (n = 14)	(1) Single oral bolus (2) Single oral dose	(1) 41 (2) 41	(1) ~ 25 (2) ~25 (geometric mean ratio 1.02, P=.86)	5-days 90-days 365-days	(1) 103 (95% CI 87–122) (2) 45 (95% CI 37–53) 5-days: geometric mean ratio = 2.31, P<.001). However, 25(OH)D concentrations were similar at 90- and 365-days between-groups	No significant different in PTH between groups at any time point

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; *values shown are median and range

Table 27(cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ or have administered intermittent bolus doses of vitamin D₃ with a frequency of >3-monthly and have measured PTH response

Reference	Participant Characteristics			Dosing regimen		Baseline PTH			PTH Response	
	Sample size for bolus	Age (years) & health status	Sex	Dose (IU)	Dosing regimen	Baseline total 25(OH)D (nmol/l)	Baseline PTH (pg/ml)	Follow-up time points	25(OH)D (nmol/l) at follow-up	PTH (pg/ml) at follow-up
<i>Single large bolus dose < 200 000IU</i>										
Rosseau et al., (2015) (245)	29	Healthy participants aged: 26 (22–60)*	M/F	100 000IU D ₃	Single bolus dose	53.8 (25.3-109.0)*	16.5 (4.5-37.4)*	1-week	100 (65-167)	15.5 (3.4-34.7)
Pekkarinen et al.,(2010) (200)	20	Community dwelling older participants from Finland aged; 74.1 (71.2-78.0) *	M/F	97333IU D ₃	Bolus dose every 4-months	57.5 (17.0-84.0)*	59 (29-164)*	1-month 2-months 3-months	Significant increases from baseline at 1-, - and 3-months	No significant change across the study period

^a PTH <.05 vs. baseline. Values are mean and SD (+/-) or mean and 95% CI unless denoted as follows; * values shown are median and range

5.2 sCa/iCa response to bolus dose vitamin D₃

A limited number of studies have also investigated the effect of a single large bolus of vitamin D₃ on sCa and iCa. Findings regarding sCa and iCa calcium are discussed below.

5.2.1 Single large bolus dose $\geq 500\ 000\text{IU}$

Most large bolus dosing studies do not show a significant effect on sCa and iCa. A significant increase in sCa was found 3-days post administration of a 600 000IU D₃ bolus in young vitamin D insufficient participants (mean sCa baseline: 2.33mmol/l [\pm 0.08] vs. mean sCa day 3: 2.38mmol/l [\pm 0.08], $P < 0.01$). However, mean values remained well within the normal range and sCa was not significantly different at other time points up to 90-days after administration (214). Mean iCa levels also did not change over the entire 180 day study period after a 600 000IU dose in younger vitamin D insufficient women (313). Tellioglu et al also report no significant change from baseline at 6- and 12-weeks after administration of a similar dose in vitamin D deficiency ambulatory older adults (212). However, one participant was reported to have a sCa level just above the upper limit of the normal range.

5.2.2 Single large bolus dose $\geq 200\ 000\text{IU}$ $\geq 300\ 000\text{IU}$

Single large bolus doses of vitamin D₃ of between 200 000IU and 300 000IU do not appear to affect sCa or iCa levels.

No hypercalcemia was reported after a 300 000IU D₃ bolus in elderly individuals with secondary hyperparathyroidism (201) or in younger vitamin D deficient rheumatology patients (215). sCa was not significantly different to baseline at 3-months (mean baseline: 2.28 [\pm 0.08] vs. mean 3-months: 2.30 [\pm 0.08], $P = .81$) after a 300 000IU D₃ bolus in vitamin D deficient community dwelling older adults. Von Restorff et al also report no change in serum calcium at 3- and 6-months after administration of a similar dose in elderly vitamin D deficient patients (216). However, they do report that two patients had hypercalcemia at 3-months, but this had corrected by 6-months (216).

sCa was not significantly different to baseline at 5-day after a 250 000IU D₃ bolus in young healthy adults and all individuals remained within the reference range at all study time points (218). After a slightly lower dose of 200 000IU in vitamin D sufficient elderly adults, sCa was not significantly different to baseline at 4-weeks (mean baseline: 2.10 [\pm 0.10] vs. mean 3-months: 2.23 [\pm 0.18], $P > .05$) (312). Also in healthy, elderly, vitamin D sufficient, iCa did not differ across the study period after a 200 000IU D₃ bolus administered every three months (311). Although, 7 out of 20 participants in this treatment group had occasional rises in iCa calcium above the normal range (up to 1.39mmol/l) (311), but it is not reported exactly when in the study period these rises in iCa occur.

5.2.3 Single large bolus dose <200 000IU

Smaller bolus doses do not appear to significantly affect sCa or iCa. Ilahi et al report that serum calcium did not rise at any time point over a 120-day period after a 100 000IU bolus dose in healthy adults (286). There was actually a biologically small (-.030 mg/dl [+/-0.38]) but statistically significant fall in sCa at 5-days post administration and there were no reports of hypercalcemia in any participant at any time point. There was also no difference in pre- and post- sCa at 7-days after a 100 000IU D₃ bolus in healthy participants and no hypercalcemia was reported (245). Similarly, in the RCT by Pekkarinen et al, mean iCa did not change across the study period after an approximate 100 000IU dose in vitamin D sufficient elderly women (200). Three women exhibited occasional mild hypercalcemia (up to 1.41mmol/l), but this was likely due to mild primary hyperparathyroidism in these participants (200).

5.3 UrCa excretion after bolus dose vitamin D₃

A limited number of vitamin D₃ bolus dosing studies have reported the effect on 24-hour urCa excretion.

5.3.1 Single large bolus dose \geq 500 000IU

A significant increase in urCa excretion was reported at 3- and 15-days after administration of a 600 000IU D₃ bolus in vitamin D insufficient young adults, but returned to baseline levels by 30-days post administration (214). Tellioglu et al report that three participants had hypercalciuria at 6-weeks and 1 participant had hypercalciuria at 12-weeks after administration of a 600 000IU D₃ bolus (212).

5.3.2 Single large bolus dose > 300 000IU

Studies that have administered smaller single bolus doses also report significant increases in urCa excretion. After a 300 000IU bolus given to older community dwelling women, there was a significant increase in urCa excretion at 3-months post-administration (mean baseline: 84 +/- 51 mg/24-hour [+/- 51] vs. mean 3-months: 118 mg/24-hour [+/- 60], $P = .003$)(217).

Twenty-four hour urCa excretion also increased significantly 1-week after a 200 000IU (mean baseline: 2.5mmol/24hr [range: 0.5-5.9] vs. mean 1-week: 4.1mmol/24hr [range: 0.4-8.2], $P < .05$) and 100 000IU (mean baseline: 2.9mmol/24hr [range: 0.6-6.9] vs. mean 1-week: 4.3mmol/24hr [range: 1.7-9.1], $P < .05$) administered to community dwelling older women (311). One participant in the 200 000IU dose group had occasional rises in 24-hour urCa above the upper limit of the normal range (10mmol/24hr), but there was also one participant in the placebo group who displayed similar occasional rises (311). Pekkarinen et al also report increases in 24-hour urCa up to 3-months after administration after a ~100 000IU D₃ bolus. In this study, one woman had a 24-hour urinary calcium excretion above the upper limit of the normal range (10mmol/24hr) at 3-months post-administration (200).

5.4 sPhos & FGF-23 response to bolus dose vitamin D₃

Few studies have reported changes in sPhos after a single bolus dose of vitamin D₃. Cipriani et al reported an immediate increase in sPhos from baseline levels after just 3-days post-administration of a 600 000IU D₃ oral bolus, but levels had fallen to reported at baseline by 15-days follow-up (214). Studies with longer durations between baseline and follow-up have not reported increases in serum phosphate (216, 217), indicating that phosphate levels may quickly return to baseline levels after a vitamin D₃ oral bolus.

FGF-23 is important in phosphate and calcium homeostasis. When sPhos levels increase, there is a concomitant increase in FGF-23 which inhibits PTH and 1, 25(OH)₂D synthesis. This serves to reduce sPhos levels. Only one study has measured FGF-23 after a single oral bolus dose of vitamin D₃. Rousseau et al reported no significant change in FGF-23 after a 100 000IU bolus in healthy young adults (Baseline: 50RU/ml [21-116] vs. 1-week: 54.3 RU/ml [23-120])(245).

5.5 Albumin and DBP

Only one has reported changes in VDBP and albumin after a single large D₃ oral bolus. There was no significant change in VDBP (Baseline: 254µg/ml [63-91] vs. 1-week: 253µg/ml [63-644]) or albumin (Baseline: 49g/l [44-56] vs. 1-week: 49g/l [42-56]) 1-week after administration (245).

5.6 Bone turnover markers

A relatively limited number of studies have reported the effects of a single bolus dose of vitamin D₃ on bone turnover. A summary of these studies can be found in **table 28**.

Very large oral bolus doses of vitamin D₃ may be associated with acute increases in bone resorption markers. After a 600 000IU bolus, increases in sCTX from baseline were observed up to 60-days post-administration (219). There was also smaller, but significant, transient changes in sCTX immediately after a smaller bolus dose of 300 000IU (219). It is not clear if these small transient changes in sCTX immediately after bolus dosing are clinically relevant, but this mechanism may help to explain the increase in fractures immediately after administration that has previously been reported (8). Interestingly, there were no changes in the bone formation marker BALP with doses of 600 000IU and 300 000IU, indicating that any transient increase in bone resorption may be too brief to be associated with changes in bone formation (219).

After lower bolus doses of 200 000IU and 100 000IU, Valimaki et al did not find a significant change in sCTX from baseline at 1-week and 3-month post administration in healthy older women (311). Other bolus dosing studies have only measured bone turnover markers after at least 1-month post-supplementation. These studies also appear to show either no change or a decrease in bone formation markers (200, 213, 217).

Table 28: A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ have measured changes in bone turnover markers

Reference	Participants	D ₃ bolus dose	Baseline Bone turnover marker	Follow up Bone turnover marker
Rossini et al., (2012) (219)	Elderly community dwelling aged: 75 +/- 3 (n= 12)	600 000IU	sCTX: 0.27 +/-0.10	1-day: 0.41 +/- 0.22 ^a 3-days: 0.41 +/- 0.22 ^a 7-days: 0.36 +/- 0.14 ^a 14-days: 0.37 +/-0.15 ^a 30-days: 0.37 +/-0.13 ^a 60-days: 0.35 +/-0.14 ^a 90-days: 0.30 +/-0.14
-	Elderly community dwelling aged: 75 +/- 3 (n= 12)	300 000IU	sCTX: 0.27 +/-0.09	1-day: 0.28 +/- 0.10 3-days: 0.30 +/- 0.09 ^a 7-days: 0.30 +/- 0.11 14-days: 0.29 +/-0.08 30-days: 0.27 +/-0.09 60-days: 0.28 +/-0.08 90-days: 0.26 +/-0.08
-	Elderly community dwelling aged: 75 +/- 3 (n= 13)	100 000IU	sCTX: 0.27 +/-0.09	1-day: 0.28 +/- 0.10 3-days: 0.29 +/- 0.10 7-days: 0.28 +/- 0.10 14-days: 0.30 +/-0.09 30-days: 0.27 +/-0.10 60-days: 0.27 +/-0.09 90-days: 0.26 +/-0.10
Bacon et al., (2009) (213)	Recently hospitalized aged: 82 +/- 7 (n=19)	500 000IU	PINP: 83 +/- 17	PINP declined over time in those with a baseline 25(OH)D<30nmol/l (P=.05) There were no changes across the study time period in those who had a baseline 25(OH)>30nmol/l
Giusti et al., (2010) (217)	Community dwelling older adults aged: 74 +/- 6 (n= 30)	300 000IU	sCTX: 0.55 +/-0.15	3-months: 0.48 +/- 0.19 ^a

^a P<.05 vs. baseline; sCTX: ng/ml; PINP: µg/l

Table 28 (cont): A summary table of intervention studies in healthy individuals that have administered a single large oral bolus dose of vitamin D₃ have measured changes in bone turnover markers

Reference	Participants	D ₃ Bolus dose	Baseline Bone turnover marker	Follow up Bone turnover marker
Valimaki et al., (2016) (311)	Healthy community dwelling aged: 75 +/- 2.9	200 000IU	sCTX: 0.52 (0.16-0.92) PINP: 38 (19-72)	1-week: 0.43 (0.11-0.77) 3-months: 0.46 (0.15-0.87) 1-week: 39 (21-64) 3-months: 43 (19-76)
-	Healthy community dwelling aged: 75 +/- 2.9	100 000IU	sCTX: 0.51 (0.15-1.33) PINP: 34 (20-113)	1-week: 0.43 (0.11-1.21) 3-months: 0.44 (0.14-1.17) 1-week: 40 (17-100) 3-months: 32 (17-82)
Pekkarinen et al., (2010) (200)	Community dwelling older participants from Finland aged; 74.1 (71.2-78.0)	~100 000IU	PINP: 51 (26-95)	A decrease in PINP reported over time (up to 4 months before administration of another dose)

^a P<.05 vs. baseline; sCTX: ng/ml; PINP: µg/l

5.7 Physical function

Studies that have investigated the association between vitamin D status and the effects of vitamin D supplementation on physical performance have yielded inconsistent findings. Some studies have demonstrated favorable effects of vitamin D supplementation physical performance, muscle strength and falls (115-118), whereas others have not demonstrated favorable effects (318).

Some studies have demonstrated a link between single large bolus doses of vitamin D (8, 9) and large repeated dose of vitamin D₃ (10) and an increase in falls and fracture. However, there have been very few studies that have investigated the effect of a single large bolus of vitamin D₃ on physical function and so the effects are unclear.

Tellioglu et al report significant increases in quadriceps muscle strength in ambulatory care home participants at 12-weeks post-administration of a single 600 000IU D₃ oral bolus (baseline: 43.4lb [+/- 9.8] vs. 12 weeks:

45.7lb [+/-9.3], $P= .039$ (212). In the same study total SPPB scores also increased significantly at 12-weeks (baseline: 7.75 [+/- 2.7] vs. 12 weeks: 8.00 [+/-2.9], $P= .039$. However, SPPB subscale scores did not change significantly after administration (212). A 300 000IU dose of vitamin D₃ given orally or IM in community dwelling adults (aged >65 years), increased quality of life scores, decreases measure of pain and improved functional mobility (236).

Other D₃ oral bolus dosing studies have not shown any effect on grip strength. In younger women, maximal grip strength did not change up to 180-days after a 600 000IU D₃ oral bolus (313). Although grip strength did increase in the study by Bacon et al after a 500 000IU D₃ bolus given to older hospitalized patients, it did so in other vitamin D treatment groups as well (monthly and 500 000IU + monthly 50 000IU) and there was no placebo group for comparison (213).

5.8 Cardiovascular outcomes

Observational studies have demonstrated an association between low 25(OH)D and cardiovascular risk factors such as, blood pressure, inflammation and dyslipidemia and overall cardiovascular mortality (119-123) but the results from intervention studies are less consistent, with some meta-analyses demonstrating a protective effect of supplementation against cardiovascular risk factors (124) and others demonstrating no protective effect (125-128).

It has been postulated that vitamin D may have a blood pressure lowering effect due to its inhibition of the renin-angiotensin-aldosterone system (238-240). Clinical studies have also demonstrated a fall in systolic and diastolic blood pressure after vitamin D supplementation (124). It is therefore possible that a single large oral bolus dose of vitamin D₃ could lead to unexpected decreases in blood pressure, which may then lead to falls. However, the effect of single large bolus doses of vitamin D₃ has not been extensively studied.

The only study to date that has investigated the effect of an oral D₃ bolus on cardiovascular outcomes, failed to demonstrate an effect of supplementation on blood pressure after a 200 000IU dose (312).

Results

5.9 Response of carrier proteins to bolus dose vitamin D₃

5.9.1 Proportional between-group differences at each time point

The response from baseline of albumin and VDBP to the three different bolus doses of vitamin D₃ (50 000IU, 150 000IU & 500 000IU) are shown in **figure 38** and **table 29**. There was no statistically significant interaction between treatment group and time point ($P = .097$), after adjustment for baseline VDBP. There was also no overall statistically significant difference in VDBP between treatment groups ($P = .941$).

There was no statistically significant interaction between treatment group and time point ($P = .872$), after adjustment for baseline albumin. There was no overall statistically significant difference in albumin between treatment groups ($P = .535$).

5.9.2 Proportional within-group changes

In the 500 000IU treatment group VDBP levels did not change over time (difference week 1 vs. baseline: 19 [95% CI: -13, 52]; difference week 4 vs. baseline: -8 [95% CI: -40, 25]; difference week 12 vs. baseline: -8 [95% CI: -40, 24]). Albumin levels also did not change over time in the 500 000IU treatment group (ratio of difference: 0.98 [95% CI: 0.96, 1.01]; ratio of difference week 4 vs. baseline: 0.98 [95% CI: 0.95, 1.00]; ratio of difference week 12 vs. baseline: 0.98 [95% CI: 0.96, 1.01]).

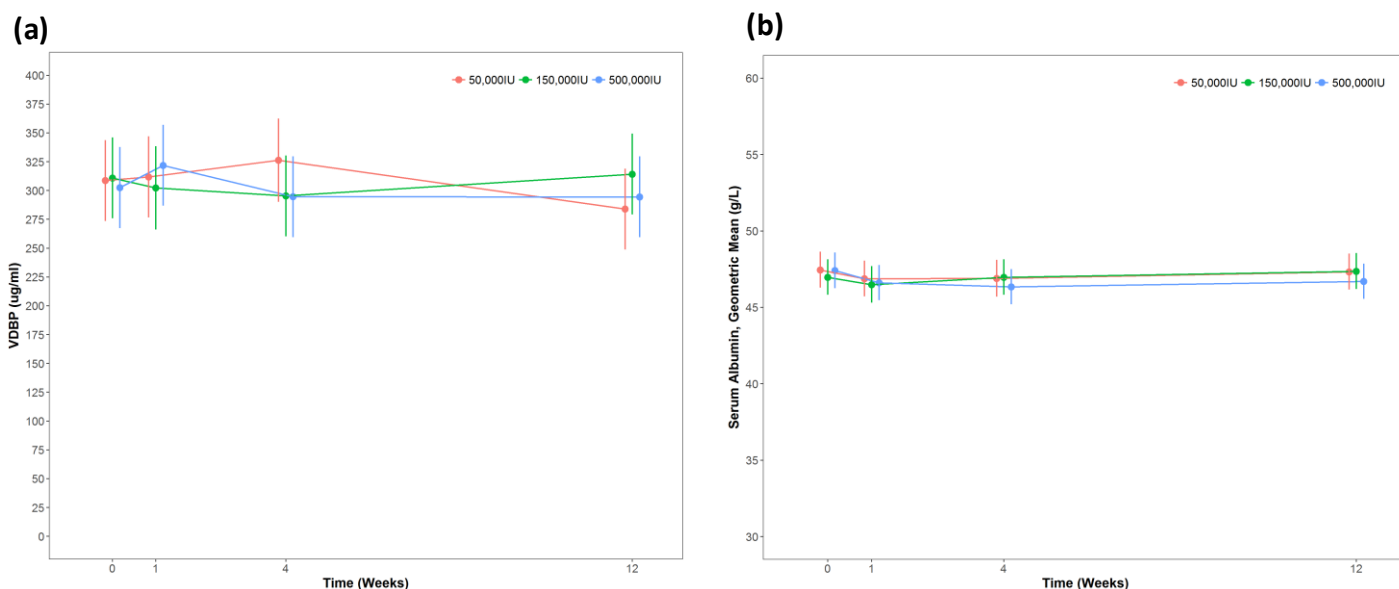
In the 150 000IU treatment group, VDBP did not change over time (week 1 vs. baseline: -9 [95% CI: -42, 25]; difference week 4 vs. baseline: -16 [95% CI: -48, 17]; difference week 12 vs. baseline: 3 [95% CI: -29, 36]). Albumin levels also did not change over time (ratio of difference: 0.99 [95% CI: 0.97, 1.01]; ratio of difference week 4 vs. baseline: 1.00 [95% CI: 0.98, 1.03]; ratio of difference week 12 vs. baseline: 1.01 [95% CI: 0.98, 1.03]).

In the 50 000IU treatment group, VDBP did not change over time (difference week 1 vs. baseline: 3 [95% CI: -29, 36]; difference week 4 vs. baseline: 18 [95% CI: -16, 51]; difference week 12 vs. baseline: -25 [95% CI: -57, 8]). Albumin levels also did not change over time (ratio of difference: 0.99 [95% CI: 0.96, 1.01]; ratio of difference week 4 vs. baseline: 0.99 [95% CI: 0.96, 1.01]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.97, 1.02]). Absolute values for VDBP and albumin at each time point are summarised in **table 29**.

5.9.3 Control group

Control group levels of albumin at baseline (Geometric mean: 48g/L [95% CI: 43, 53]) and week 12 (Geometric mean: 47g/L [95% CI: 45, 49]) were similar ($t(26) = 1.197$, $P = .242$) and control group levels of VDBP at baseline (Geometric mean: 305µg/ml [95% CI: 284- 326]) and week 12 (Geometric mean: 309µg/ml [95% CI: 287, 331]) were similar ($t(26) = -0.431$, $P = .670$).

Figure 38: Changes in (a) VDBP and; (b) albumin by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values for VDBP are arithmetic means and values for albumin are geometric means. Bars represent 95% Confidence Intervals.

Table 29: Mean VDBP and albumin at each time point.

Treatment Group	VDBP (µg/ml)				Albumin (g/L)			
	Visit (Weeks)							
	0	1	4	12	0	1	4	12
50 000IU	308 (273, 343)	311 (276, 346)	326 (290, 362)	283 (248, 318)	47 (46, 49)	47 (46, 48)	47 (46, 48)	47 (46, 49)
150 000IU	310 (275, 345)	302 (266, 338)	295 (260, 330)	314 (279, 349)	47 (46, 48)	47 (45, 48)	47 (46, 48)	47 (46, 49)
500 000IU	303 (267, 338)	322 (287, 357)	295 (260, 330)	295 (259, 330)	47 (46, 49)	46 (46, 48)	46 (45, 48)	47 (46, 48)

Values for VDBP are arithmetic means and 95% CIs and values for albumin are geometric means and 95% CIs.

5.10 Response of PTH to bolus dose supplementation

5.10.1 Proportional between-group differences at each time point

The response of PTH to the three different bolus doses of cholecalciferol is shown in **figure 39** and **table 30**.

There was a statistically significant interaction between treatment group and time point ($P = .038$) suggesting that the difference in PTH between treatment groups changes over time. However, post-hoc analysis indicated that none of the differences between treatment groups were statistically significant.

5.10.2 Proportional within-group changes

In the 500 000IU treatment group at week 1, PTH values were not different to baseline (ratio of difference week 1 vs. baseline: 0.85 [95% CI: 0.71, 1.01]) but at week 4 levels were significantly lower than baseline (ratio of difference week 4 vs. baseline: 0.83 [95% CI: 0.70, 0.99]). By week 12, PTH levels were back at baseline levels (ratio of difference week 12 vs. baseline: 0.96 [95% CI: 0.81, 1.15]).

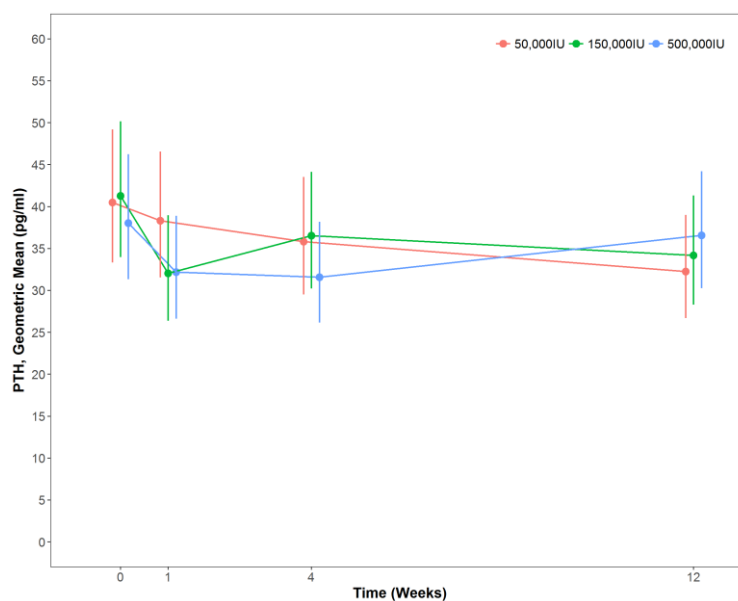
In the 150 000IU treatment group at week 1, PTH had fallen from baseline (ratio of difference week 1 vs. baseline: 0.78 [95% CI: 0.65, 0.93]). PTH had increased by week 4 and was not different to baseline (ratio of difference week 4 vs. baseline: 0.88 [95% CI: 0.74, 1.06]), but was lower than baseline at week 12 (ratio of difference week 12 vs. baseline: 0.83 [95% CI: 0.69, 0.99]).

In the 50 000IU treatment group at week 1, PTH was similar to baseline (ratio of difference week 1 vs. baseline: 0.95 [95% CI: 0.79, 1.14]) and remained similar at week 4 (ratio of difference week 4 vs. baseline: 0.88 [95% CI: 0.74, 1.06]). By week 12, PTH had fallen to below baseline levels (ratio of difference week 12 vs. baseline: 0.80 [95% CI: 0.67, 0.95]). Absolute values for PTH at each time point are summarised in **table 30**.

5.10.3 Control group

Control group levels of PTH at baseline (Geometric Mean: 31.5pg/ml [95% CI: 27.5, 36.2]) were not significantly different to week 12 (Geometric Mean: 32.6pg/ml [95% CI: 28.6, 37.2]) ($t(26) = -.565$, $P = .577$).

Figure 39: Changes in PTH by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% Confidence Intervals.

Table 30: Mean PTH (pg/ml) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	40.5 (33.3, 49.2)	38.3 (31.5, 46.5)	35.8 (29.5, 43.5)	32.3 (26.7, 39.0)
150 000IU	41.3 (34.0, 50.1)	32.0 (26.4, 38.9)	36.5 (30.2, 44.1)	34.2 (28.3, 41.3)
500 000IU	38.0 (31.3, 46.2)	32.2 (26.6, 38.9)	31.6 (26.1, 38.2)	36.6 (30.2, 44.2)

Values are geometric means and 95% CIs.

5.11 Response of sCa and iCa to bolus dose supplementation

5.11.1 Proportional between-group differences at each time point

The response of adjusted sCa and iCa to the three bolus doses of cholecalciferol are shown in **figure 40** and **table 31**. There was no statistically significant interaction between treatment group and time point ($P = .989$) and there was no overall significant difference between groups at any time point for sCa ($P = .626$), after adjustment for baseline values. For iCa, there was no significant interaction between treatment group and time point ($P = .163$), after adjustment for baseline values. There was also no overall significant difference between treatment groups at any time point ($P = .147$).

5.11.2 Proportional within-group changes

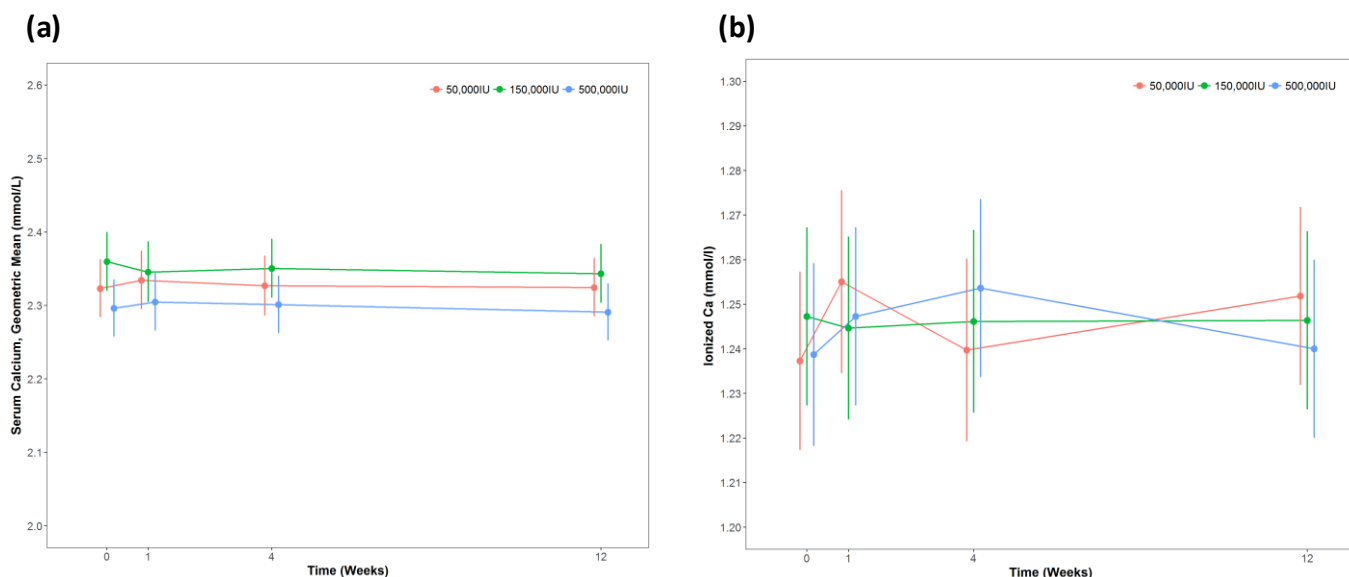
In the 500 000IU treatment group at week 1, sCa was similar to baseline at all time points (ratio of difference week 1 vs. baseline: 1.00 [95% CI: 0.99, 1.02]; ratio of difference week 4 vs. baseline: 1.00 [95% CI: 0.99, 1.02]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.98, 1.01]). sCa was also similar to baseline at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 0.99 [95% CI: 0.98, 1.01]; ratio of difference week 4 vs. baseline: 1.00 [95% CI: 0.98, 1.01]; ratio of difference week 12 vs. baseline: 0.99 [95% CI: 0.98, 1.01]) and the 50 000IU treatment group (ratio of difference week 1 vs. baseline: 1.00 [95% CI: 0.99, 1.02]; ratio of difference week 4 vs. baseline: 1.00 [95% CI: 0.98, 1.02]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.98, 1.02]).

In the 500 000IU treatment group at week 1, iCa was similar to baseline at all time points (difference week 1 vs. baseline: 0.009 [95% CI: -0.010, 0.027]; difference week 4 vs. baseline: 0.015 [95% CI: -0.003, 0.033]; ratio of difference week 12 vs. baseline: 0.001 [95% CI: -0.017, 0.019]). iCa was also similar to baseline at all time points in the 150 000IU treatment group (difference week 1 vs. baseline: -0.003 [95% CI: -0.021, 0.015]; difference week 4 vs. baseline: -0.001 [95% CI: -0.019, 0.017]; difference week 12 vs. baseline: -0.001 [95% CI: -0.019, 0.017]) and the 50 000IU treatment group (difference week 1 vs. baseline: 0.018 [95% CI: -0.0003, 0.036]; difference week 4 vs. baseline: 0.002 [95% CI: -0.016, 0.021]; difference week 12 vs. baseline: 0.015 [95% CI: -0.003, 0.032]). Absolute values for sCa and iCa at each time point are summarised in **table 31**.

5.11.3 Control group

Control group levels of sCa at baseline (Geometric mean: 2.33nmol/l [95% CI: 2.30, 2.36]) were not significantly different to week 12 (Geometric mean: 2.32nmol/l [95% CI: 2.29, 2.36]) ($t(26) = -.548, P = .588$). Control group levels of iCa also did not differ from baseline (mean: 1.25nmol/l [95% CI: 1.23, 1.26]) at the end of the study period (mean: 1.25nmol/l [95% CI: 1.23, 1.26]) ($t(21) = .000, P = 1.00$).

Figure 40: Changes in (a) adjusted serum calcium and; (b) ionized calcium by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Dots are geometric means for sCa and arithmetic means for iCa. Bars represent 95% Confidence Intervals.

Table 31: Mean adjusted serum calcium and ionized calcium at each time point.

Treatment Group	sCa (nmol/l)				iCa (nmol/l)			
	Visit (Weeks)							
	0	1	4	12	0	1	4	12
50 000IU	2.32 (2.28, 2.36)	2.33 (2.29, 2.37)	2.33 (2.29, 2.37)	2.32 (2.29, 2.36)	1.23 (1.22, 1.26)	1.26 (1.23, 1.28)	1.24 (1.22, 1.26)	1.25 (1.23, 1.27)
150 000IU	2.36 (2.32, 2.40)	2.35 (2.30, 2.39)	2.35 (2.31, 2.39)	2.34 (2.30, 2.38)	1.25 (1.23, 1.27)	1.25 (1.22, 1.27)	1.25 (1.23, 1.27)	1.25 (1.23, 1.27)
500 000IU	2.30 (2.26, 2.34)	2.30 (2.27, 2.34)	2.30 (2.26, 2.34)	2.29 (2.25, 2.33)	1.24 (1.22, 1.26)	1.25 (1.23, 1.27)	1.25 (1.23, 1.27)	1.24 (1.22, 1.26)

Values for sCa are geometric means and 95% CIs and values for iCa are arithmetic means and 95% CIs.

5.12 Response of sCr & sPhos to bolus dose supplementation

5.12.1 Proportional between-group differences at each time point

The response of sCr and serum phosphate sPhos to the three bolus doses of vitamin D₃ are shown in **figure 41** and **table 32**. There was no statistically significant interaction between treatment group and time point ($P = .907$) on sCr, after adjustment for baseline values. There was also no overall significant difference between groups at any time point ($P = .452$). For sPhos, there was no significant interaction between treatment group and time point ($P = .469$), after adjustment for baseline values. There was also no significant difference between treatment groups at any time point ($P = .156$).

5.12.2 Proportional within-group changes

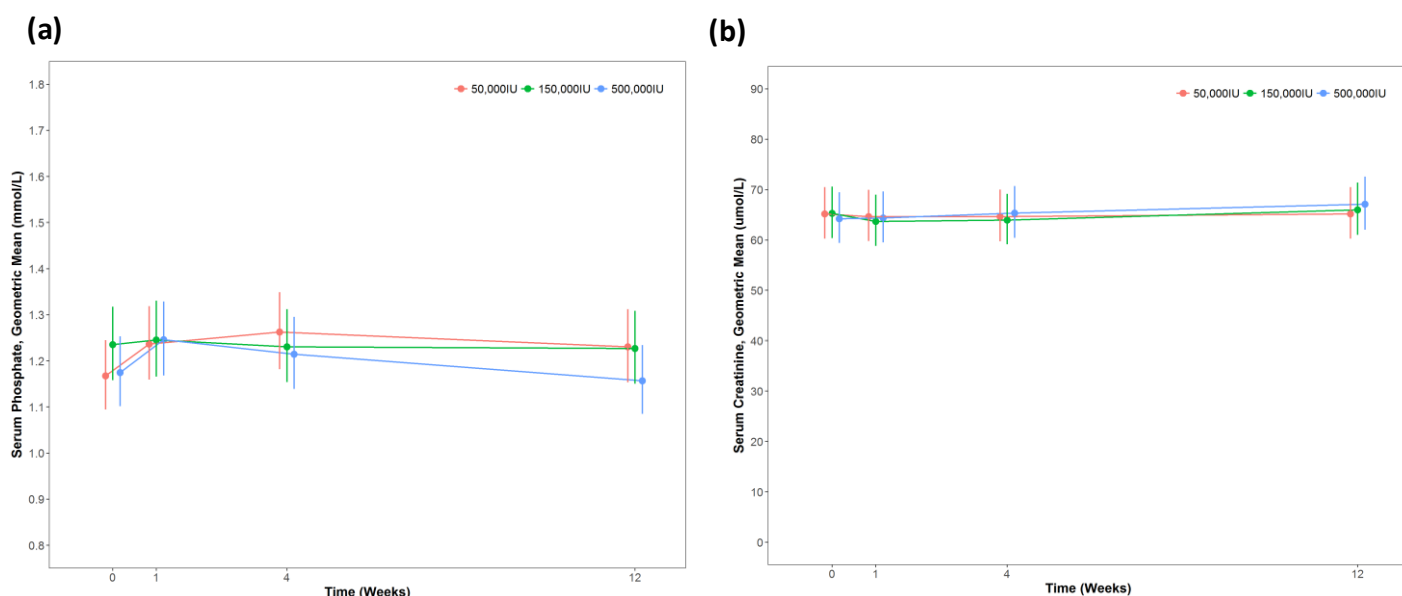
In the 500 000IU treatment group at week 1, sCr was similar to baseline at all time points (ratio of difference week 1 vs. baseline: 1.00 [95% CI: 0.95, 1.05]; ratio of difference week 4 vs. baseline: 1.02 [95% CI: 0.97, 1.07]; ratio of difference week 12 vs. baseline: 1.04 [95% CI: 0.99, 1.10]). sCr was also similar to baseline at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 0.98 [95% CI: 0.93, 1.03]; ratio of difference week 4 vs. baseline: 0.98 [95% CI: 0.93, 1.03]; ratio of difference week 12 vs. baseline: 1.01 [95% CI: 0.96, 1.06]) and the 50 000IU treatment group (ratio of difference week 1 vs. baseline: 0.99 [95% CI: 0.94, 1.04]; ratio of difference week 4 vs. baseline: 0.99 [95% CI: 0.94, 1.04]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.95, 1.05]).

In the 500 000IU treatment group at week 1, sPhos was similar to baseline at all time points (ratio of difference week 1 vs. baseline: 1.06 [95% CI: 1.00, 1.12]; ratio of difference week 4 vs. baseline: 1.03 [95% CI: 0.98, 1.09]; ratio of difference week 12 vs. baseline: 0.98 [95% CI: 0.93, 1.04]). sPh was also similar to baseline at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 1.01 [95% CI: 0.95, 1.07]; ratio of difference week 4 vs. baseline: 1.00 [95% CI: 0.94, 1.05]; ratio of difference week 12 vs. baseline: 1.00 [95% CI: 0.94, 1.05]) and the 50 000IU treatment group (ratio of difference week 1 vs. baseline: 1.06 [95% CI: 1.00, 1.12]; ratio of difference week 4 vs. baseline: 1.08 [95% CI: 1.00, 1.12]; ratio of difference week 12 vs. baseline: 1.05 [95% CI: 1.00, 1.11]). Absolute values for sCr and sPhos at each time point are summarised in **table 32**.

5.12.3 Control group

Control group levels of sCr at baseline (Geometric mean: 66 $\mu\text{mol/l}$ [95% CI: 57, 76]) were not significantly different to week 12 (Geometric mean: 67 $\mu\text{mol/l}$ [95% CI: 59, 75]) ($t(26) = -.396$, $P = .696$). Control group levels of sPhos also did not differ from baseline (Geometric mean: 1.16 mmol/l [95% CI: 1.08, 1.26]) at the end of the study period (Geometric mean: 1.18 nmol/l [95% CI: 1.08, 1.28]) ($t(26) = -.588$, $P = .561$).

Figure 41: Changes in (a) serum phosphate and; (b) serum creatinine by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% Confidence Intervals

Table 32: Mean serum creatinine and serum phosphate at each time point.

Treatment Group	sCr (nmol/l)				sPhos (nmol/l)			
	Visit (Weeks)							
	0	1	4	12	0	1	4	12
50 000IU	65.1 (60.2, 70.5)	64.6 (59.7, 69.9)	64.6 (59.7, 70.0)	65.1 (60.2, 70.5)	1.17 (1.09, 1.24)	1.24 (1.16, 1.32)	1.26 (1.18, 1.35)	1.23 (1.15, 1.31)
150 000IU	65.3 (60.3, 70.6)	63.7 (58.8, 68.9)	63.9 (59.1, 69.1)	66.0 (61.0, 71.4)	1.24 (1.16, 1.32)	1.25 (1.17, 1.33)	1.23 (1.15, 1.31)	1.23 (1.15, 1.31)
500 000IU	64.2 (59.4, 69.4)	64.4 (59.5, 69.6)	65.3 (60.4, 70.6)	67.1 (62.0, 72.6)	1.17 (1.10, 1.25)	1.25 (1.17, 1.33)	1.21 (1.14, 1.30)	1.16 (1.08, 1.23)

Values are geometric means and 95% CIs.

5.13 Response of FGF-23 to bolus dose supplementation

5.13.1 Proportional between-group differences at each time point

The response of FGF-23 to the three different bolus doses of vitamin D₃ is shown in **figure 42** and **table 33**.

There was no statistically significant interaction between treatment group and time point ($P = .110$), after adjustment for baseline FGF-23. There was also no overall significant difference between groups at any time point ($P = .918$).

5.13.2 Proportional within-group changes

In the 500 000IU treatment group at week 1, FGF-23 values increased significantly from baseline (ratio of difference week 1 vs. baseline: 1.25 [95% CI: 1.03, 1.51]). FGF-23 returned to baseline levels by week 4 (ratio of difference week 4 vs. baseline: 1.04 [95% CI: 0.86, 1.26]) and remained at similar levels at week 12 (ratio of difference week 12 vs. baseline: 0.91 [95% CI: 0.75, 1.10]).

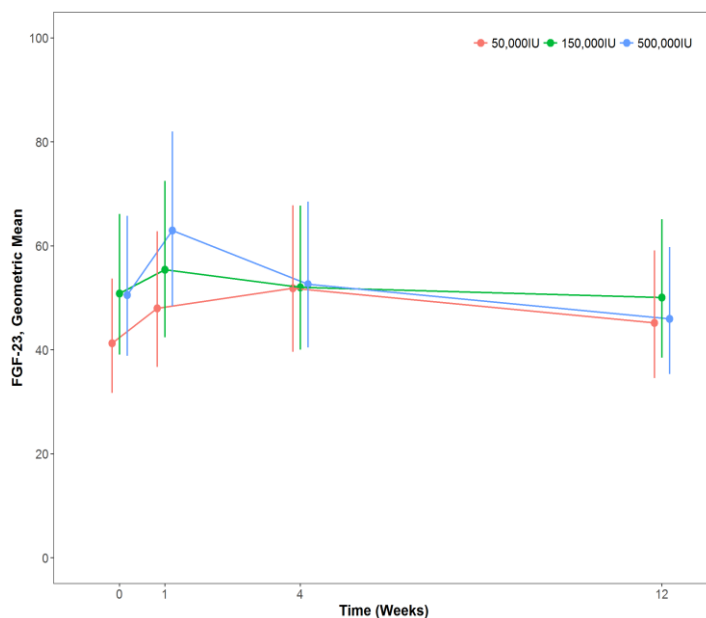
FGF-23 was similar to baseline at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 1.09 [95% CI: 0.89, 1.33]; ratio of difference week 4 vs. baseline: 1.02 [95% CI: 0.84, 1.24]; ratio of difference week 12 vs. baseline: 0.98 [95% CI: 0.81, 1.20]).

In the 50 000IU treatment group at week 1, FGF-23 was similar to baseline at week 1 (ratio of difference week 1 vs. baseline: 1.16 [95% CI: 0.95, 1.42]). FGF-23 increased significantly at week 4 (ratio of difference week 4 vs. baseline: 1.26 [95% CI: 1.03, 1.53]), but had fallen again by week 12 (ratio of difference week 12 vs. baseline: 1.09 [95% CI: 0.67, 0.95]). Absolute values for FGF-23 at each time point are summarised in **table 33**.

5.13.3 Control group

Control group levels of FGF-23 at baseline (Geometric mean: 43pg/ml [95% CI: 32, 58]) were not significantly different to week 12 (Geometric mean: 46pg/ml [95% CI: 34, 63]) ($t(26) = -1.190$, $P = .245$).

Figure 42: Changes in FGF-23 by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% Confidence Intervals.

Table 33: Mean FGF-23 (pg/ml) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	41.3 (31.7, 53.7)	48.0 (36.7, 62.7)	51.8 (39.6, 67.8)	45.2 (34.5, 59.1)
150 000IU	50.8 (39.0, 66.1)	55.4 (42.4, 72.5)	52.0 (40.0, 67.7)	50.0 (38.4, 65.1)
500 000IU	50.5 (38.8, 65.7)	63.0 (48.4, 81.9)	52.6 (40.4, 68.5)	45.9 (35.4, 59.8)

Values are geometric means and 95% CIs.

5.14 Response of 24-hour urCa/urCr to bolus dose supplementation

5.14.1 Proportional between-group differences at each time point

Changes in urCa:urCr is shown in **figure 43** and **table 34**. There was no statistically significant interaction between treatment group and time point on uCa:urCr ($P = .606$), after adjustment for baseline urCa:urCr. There was also no overall significant difference between treatment groups at any time point ($P = .057$).

5.14.2 Proportional within-group changes

In the 500 000IU treatment group at week 1, urCa:urCr increased significantly from baseline (difference week 1 vs. baseline: 0.14 [95% CI: 0.06, 0.22]) and remained similar at week 4 (difference week 4 vs. baseline: 0.09 [95% CI: 0.02, 0.17]). By week 12 urCa:urCr had fallen and was similar to baseline (difference week 12 vs. baseline: 0.06 [95% CI: -0.02, 0.13]).

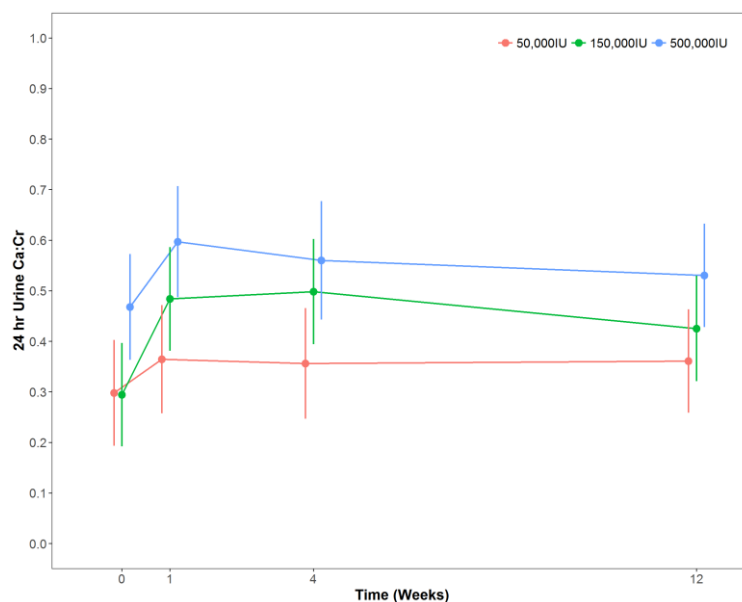
In the 150 000IU treatment group, the urCa:urCr was higher than baseline at all time points. At week 1, the urCa:urCr increased from baseline (difference week 1 vs. baseline: 0.19 [95% CI: 0.12, 0.26]) and remained at similar levels at week 4 (difference week 4 vs. baseline: 0.20 [95% CI: 0.13, 0.28]) and week 12 (difference week 12 vs. baseline: 0.13 [95% CI: 0.06, 0.20]).

In the 50 000IU treatment group, the urCa:urCr was similar to baseline at all time points (difference week 1 vs. baseline: 0.07 [95% CI: -0.01, 0.14]; difference week 4 vs. baseline: 0.07 [95% CI: -0.01, 0.14]; difference week 12 vs. baseline: 0.06 [95% CI: -0.01, 0.14]). Values for the urCa:urCr at each time point are summarised in **table 34**.

5.14.3 Control group

Control group urCa:urCr at baseline (mean: 0.36 [95% CI: 0.28, 0.44]) was not significantly different to week 12 (Mean: 0.36 [95% CI: 0.28, 0.43]) ($t(21) = .138, P = .892$).

Figure 43: Changes in the urCa:urCr by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 34: Mean urCa:urCr at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.30 (0.19, 0.40)	0.36 (0.26, 0.47)	0.36 (0.26, 0.47)	0.36 (0.26, 0.46)
150 000IU	0.29 (0.19, 0.40)	0.48 (0.38, 0.59)	0.50 (0.39, 0.60)	0.43 (0.32, 0.53)
500 000IU	0.47 (0.37, 0.58)	0.61 (0.51, 0.72)	0.57 (0.46, 0.67)	0.53 (0.43, 0.63)

Values are arithmetic means and 95% CIs

5.15 Response of bone turnover markers to bolus dose supplementation

5.15.1 Proportional between-group differences at each time point

Profiles for PINP, OC and CTX in response to the different bolus doses of vitamin D₃ are shown in **figure 44 & tables 35, 36 and 37**. There was no statistically significant interaction between treatment group and time point for any of the markers of bone turnover after adjustments for baseline levels (PINP: $P = .435$; OC: $P = .410$; CTX: $P = .733$). There was also no overall significant difference between treatment groups for any of the markers of bone turnover at any time point (PINP: $P = .122$; OC: $P = .111$; CTX: $P = .112$).

5.15.2 Proportional within-group changes

PINP

In the 500 000IU treatment group at week 1, PINP increased significantly from baseline (ratio of difference week 1 vs. baseline: 1.09 [95% CI: 1.00, 1.19]) and remained at similar levels at week 4 (ratio of difference week 4 vs. baseline: 1.15 [95% CI: 1.05, 1.26]). PINP returned to baseline levels by week 12 (ratio of difference week 12 vs. baseline: 0.99 [95% CI: 0.90, 1.08]).

PINP was similar to baseline levels at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 1.05 [95% CI: 0.95, 1.15]; ratio of difference week 4 vs. baseline: 1.04 [95% CI: 0.95, 1.13]; ratio of difference week 12 vs. baseline: 0.97 [95% CI: 0.89, 1.07]) and the 50 000IU treatment group (ratio of difference week 1 vs. baseline: 1.04 [95% CI: 0.95, 1.14]; ratio of difference week 4 vs. baseline: 0.99 [95% CI: 0.90, 1.08]; ratio of difference week 12 vs. baseline: 0.94 [95% CI: 0.86, 1.03]). Absolute values for PINP at each time point are summarised in **table 35**.

Osteocalcin

In the 500 000IU treatment group at week 1, OC increased significantly from baseline (ratio of difference week 1 vs. baseline: 1.23 [95% CI: 1.05, 1.45]). OC had fallen by week 4 and was similar to baseline (ratio of difference week 4 vs. baseline: 1.11 [95% CI: 0.94, 1.31]) and remained at similar levels at week 12 (ratio of difference week 12 vs. baseline: 0.95 [95% CI: 0.80, 1.12]).

OC was similar to baseline levels at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 1.04 [95% CI: 0.88, 1.23]; ratio of difference week 4 vs. baseline: 1.04 [95% CI: 0.88, 1.23]; ratio of difference week 12 vs. baseline: 0.95 [95% CI: 0.80, 1.13]).

In the 50 000IU treatment group, OC was similar to baseline at week 1 and week 4 (ratio of difference week 1 vs. baseline: 1.10 [95% CI: 0.93, 1.30]; ratio of difference week 4 vs. baseline: 0.91 [95% CI: 0.77, 1.08]), but

had fallen to slightly below baseline levels at week 12 (ratio of difference week 12 vs. baseline: 0.79 [95% CI: 0.67, 0.93]). Absolute values for OC at each time point are summarised in **table 36**.

CTX

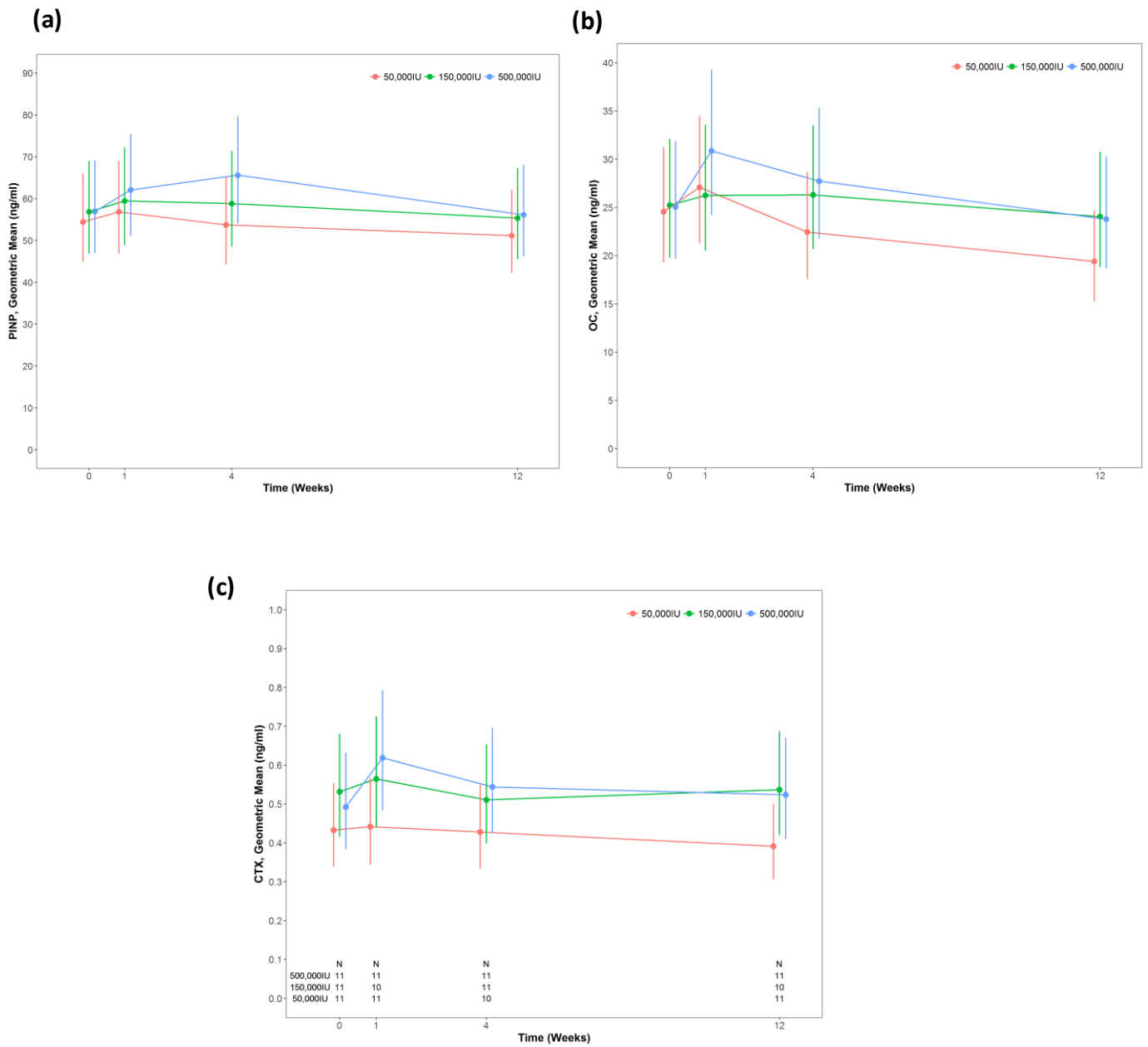
In the 500 000IU treatment group at week 1, CTX increased significantly from baseline (ratio of difference week 1 vs. baseline: 1.26 [95% CI: 1.08, 1.47]). CTX had fallen slightly by week 4 and was similar to baseline values (ratio of difference week 4 vs. baseline: 1.10 [95% CI: 0.94, 1.29]) and remained at similar to baseline at week 12 (ratio of difference week 12 vs. baseline: 1.06 [95% CI: 0.91, 1.24]).

CTX was similar to baseline values at all time points in the 150 000IU treatment group (ratio of difference week 1 vs. baseline: 1.06 [95% CI: 0.91, 1.24]; ratio of difference week 4 vs. baseline: 0.96 [95% CI: 0.83, 1.12]; ratio of difference week 12 vs. baseline: 0.1.01 [95% CI: 0.87, 1.17]) and the 50 000IU treatment group (ratio of difference week 1 vs. baseline: 1.02 [95% CI: 0.87, 1.19]; ratio of difference week 4 vs. baseline: 0.99 [95% CI: 0.85, 1.16]; ratio of difference week 12 vs. baseline: 0.90 [95% CI: 0.78, 1.05]). Absolute values for CTX at each time point are summarised in **table 37**.

5.15.3 Control group

Control group levels of PINP at baseline (Geometric mean: 52.2 ng/ml [95% CI: 38.3, 71.1]) were not significantly different to week 12 (Geometric mean: 53.8 μ mol/l [95% CI: 41.6, 69.5]) ($t(26) = -1.058$, $P = .300$). There was also no significant difference in control group CTX levels at baseline (Geometric mean: 0.44 ng/ml [95% CI: 0.33, 0.59]) compared to week 12 (Geometric mean: 0.41 μ mol/l [95% CI: 0.30, 0.57]) ($t(26) = .970$, $P = .341$). However, control group levels of OC at baseline (Geometric mean: 23.7 ng/ml [95% CI: 16.6, 33.9]) were slightly higher than at week 12 (Geometric mean: 19.9nmol/l [95% CI: 12.8, 31.1]) ($t(26) = 2.082$, $P = .009$).

Figure 44: Changes in (a) PINP; (b) OC and; (c) CTX by treatment group in response to supplementation.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are geometric means and bars represent 95% Confidence Intervals.

Table 35: Mean serum PINP (ng/ml) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	54.4 (44.8, 66.0)	56.8 (46.8, 68.9)	53.7 (44.2, 65.3)	51.1 (42.1, 62.1)
150 000IU	56.8 (46.8, 68.9)	59.4 (48.9, 72.2)	58.8 (48.5, 71.4)	55.3 (45.5, 67.2)
500 000IU	56.9 (46.9, 69.1)	62.1 (51.1, 75.3)	65.6 (54.1, 79.6)	56.1 (46.2, 68.1)

Values are geometric means and 95% CIs.

Table 36: Mean serum OC (ng/ml) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	24.6 (19.3, 31.3)	27.1 (21.3, 34.5)	22.5 (17.6, 28.7)	19.4 (15.2, 24.7)
150 000IU	25.2 (19.8, 22.1)	26.3 (20.5, 33.5)	26.3 (20.7, 33.5)	24.0 (18.8, 30.7)
500 000IU	25.0 (19.7, 31.9)	30.9 (24.2, 39.3)	27.7 (21.8, 35.3)	23.8 (18.7, 30.3)

Values are geometric means and 95% CIs.

Table 37: Mean serum CTX (ng/ml) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.43 (0.34, 0.55)	0.44 (0.34, 0.57)	0.43 (0.33, 0.55)	0.39 (0.31, 0.50)
150 000IU	0.53 (0.42, 0.68)	0.56 (0.44, 0.72)	0.51 (0.40, 0.65)	0.54 (0.42, 0.69)
500 000IU	0.49 (0.38, 0.63)	0.62 (0.48, 0.79)	0.54 (0.42, 0.70)	0.52 (0.41, 0.67)

Values are geometric means and 95% CIs.

5.16 Response of muscle strength and muscle function parameters to bolus dose supplementation

5.16.1 Proportional between-group differences at each time point

SPPB

SPPB scores in response to the bolus doses of vitamin D₃ are shown in **figure 45** and **table 38**. There was no statistically significant interaction between treatment group and study time point ($P = .676$), after adjustment for baseline SPPB score. There was also no overall statistically significant difference in SPPB scores between treatment groups ($P = .287$).

Individual components of the SPPB

Scores from the stand-alone components of the SPPB (repeated chair stand, narrow walk and balance test) at each time point are shown in **figure 46** and **table 39**. There was no significant interaction between treatment group and study time point for any of the stand-alone components (repeated chair stand: $P = .492$; narrow walk: $P = .507$; balance test: $P = .908$). There was also no overall significant difference between treatment groups for any of the individual components (repeated chair stand: $P = .159$; narrow walk: $P = .082$; balance test: $P = .318$).

5.16.2 Proportional within-group changes

SPPB

SPPB scores did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.1 [95% CI: -0.6, 0.8]; difference week 4 vs. baseline: -0.8 [95% CI: -1.5, 0.003]; difference week 12 vs. baseline: 0.0 [95% CI: -0.7, 0.7]), the 150 000IU treatment group (difference week 1 vs. baseline: 0.1 [95% CI: -0.6, 0.8]; difference week 4 vs. baseline: -0.1 [95% CI: -0.8, 0.6]; difference week 12 vs. baseline: 0.1 [95% CI: -0.6, 0.8]) and the 50 000IU treatment group (difference week 1 vs. baseline: 0.5 [95% CI: -0.3, 1.2]; difference week 4 vs. baseline: 0.3 [95% CI: -0.5, 1.0]; difference week 12 vs. baseline: 0.4 [95% CI: -0.4, 1.1]). Absolute SPPB scores at each time point are summarised in **table 38**.

Individual components of the SPPB

Repeated Chair Stand Test scores did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.1 [95% CI: -0.3, 0.5]; difference week 4 vs. baseline: -0.0 [95% CI: -0.4, 0.4]; difference week 12 vs. baseline: -0.1 [95% CI: -0.5, 0.3]) and the 150 000IU treatment group (difference week 1 vs. baseline: 0.3 [95% CI: -0.1, 0.6]; difference week 4 vs. baseline: 0.2 [95% CI: -0.2, 0.6]; difference week 12 vs. baseline: 0.5 [95% CI: -0.1, 0.8]). There was a small but significant increase in the repeated chair stand score in the 50 000IU treatment group at week 1 (difference week 1 vs. baseline: 0.4 [95% CI: 0.1, 0.8]) and the scores remained similar at week 4 (difference week 4 vs. baseline: 0.4 [95% CI: 0.1, 0.8]) and week 12 (difference week 12 vs. baseline: 0.4 [95% CI: 0.1, 0.8]).

Narrow Walk Test scores did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.0 [95% CI: -0.3, 0.3]; difference week 4 vs. baseline: 0.0 [95% CI: -0.3, 0.3]; difference week 12 vs. baseline: 0.1 [95% CI: -0.2, 0.3]), the 150 000IU treatment group (difference week 1 vs. baseline: -0.1 [95% CI: -0.3, 0.2]; difference week 4 vs. baseline: -0.2 [95% CI: -0.4, 0.1]; difference week 12 vs. baseline: -0.3 [95% CI: -0.5, 0.02]) and the 50 000IU treatment group (difference week 1 vs. baseline: 0.2 [95% CI: -0.2, 0.4]; difference week 4 vs. baseline: 0.0 [95% CI: -0.3, 0.3]; difference week 12 vs. baseline: 0.1 [95% CI: -0.2, 0.3]). Absolute values for repeated chair stand and narrow walk scores at each time point are summarised in **table 39**.

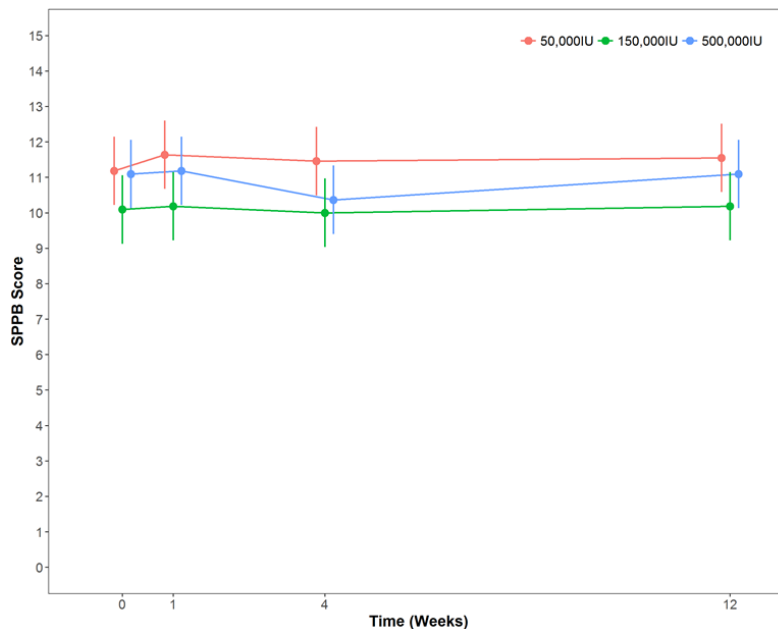
There was no variability in two of the groups in balance scores (everybody scored 4) and so there is no model.

5.16.3 Control group

Control group SPPB scores at baseline (mean: 10.7 [95% CI: 10.2, 11.2]) were not significantly different to week 12 (mean: 10.9 [95% CI: 10.5, 11.4]) ($t(26) = -1.000, P = .327$). Control group repeated chair stand scores at baseline (mean: 2.9 [95% CI: 2.5, 3.3]) were not significantly different to week 12 (mean: 3.1 [95% CI: 2.7, 3.5]) ($t(26) = -1.308, P = .202$). Control group narrow walk scores at baseline (mean: 3.9 [95% CI: 3.8, 4.0]) were not

significantly different to week 12 (mean: 3.9 [95% CI: 3.7, 4.0]) ($t(26) = .000, P = .1.00$). Control group balance test scores at baseline (mean: 3.8 [95% CI: 3.6, 4.0]) were not significantly different to week 12 (mean = 3.7 [95% CI: 3.3, 4.0]) ($t(26) = .000, P = .1.00$).

Figure 45: Change in SPPB score by treatment group.



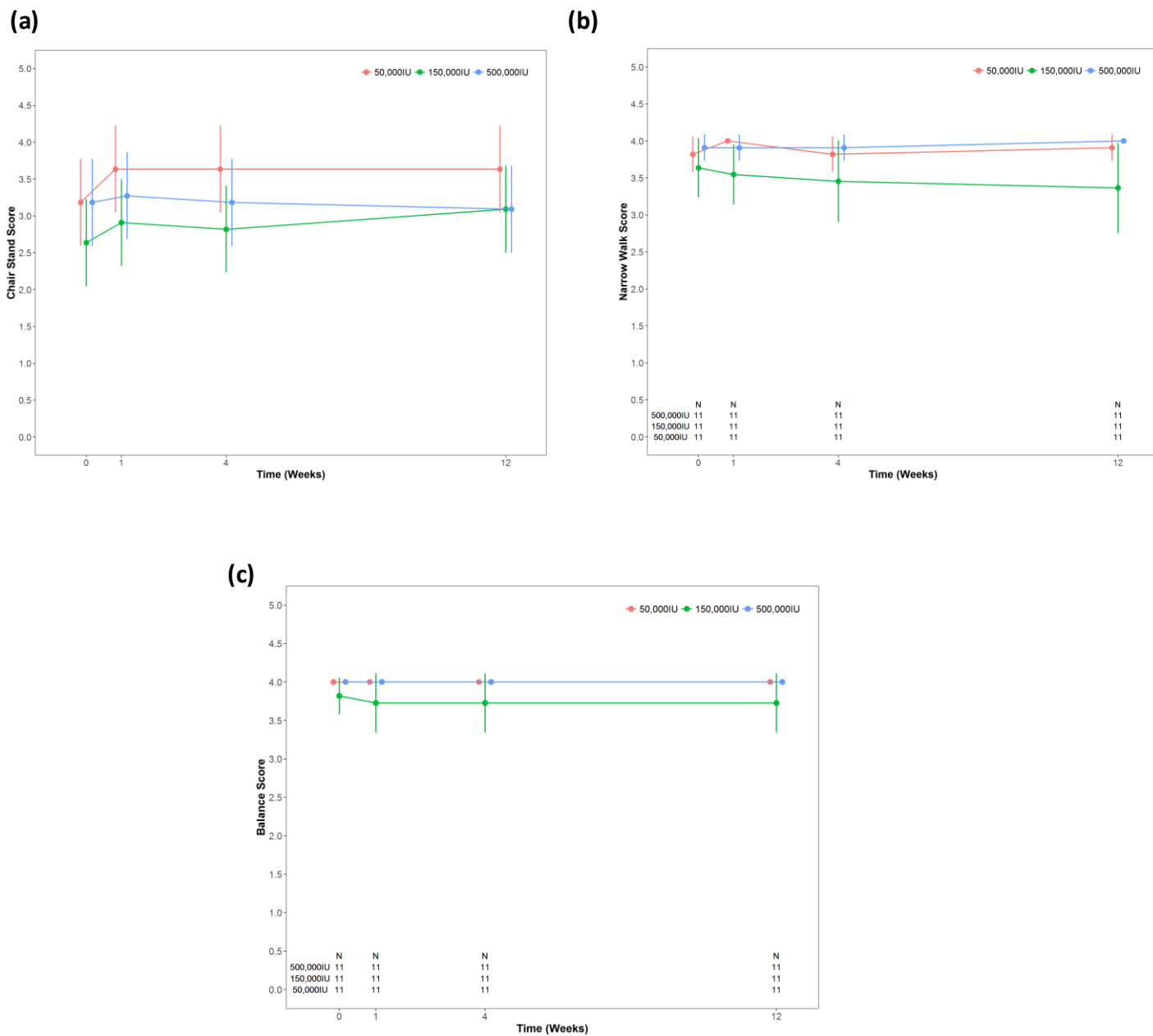
Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 38: Mean overall SPPB scores at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	11.2 (10.2, 12.1)	11.6 (10.7, 12.6)	11.5 (10.5, 12.4)	11.5 (10.6, 12.5)
150 000IU	10.1 (9.1, 11.1)	10.2 (9.2, 11.1)	10.0 (9.0, 11.0)	10.2 (9.2, 11.1)
500 000IU	11.1 (10.1, 12.1)	11.2 (10.2, 12.1)	10.4 (9.4, 11.3)	11.1 (10.1, 12.1)

Values are arithmetic means and 95% CIs

Figure 46: Changes in (a) repeated chair stand scores; (b) narrow walk test scores and; (c) balance test scores by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 39: Mean repeated chair stand and narrow walk test scores at each time point.

Treatment Group	Repeated Chair Stand				Narrow Walk			
	Visit (Weeks)							
	0	1	4	12	0	1	4	12
50 000IU	3.2 (2.6, 3.8)	3.6 (3.0, 4.2)	3.6 (3.0, 4.2)	3.6 (3.0, 4.2)	3.8 (3.5, 4.1)	4.0 (3.7, 4.3)	3.8 (3.5, 4.1)	3.9 (3.6, 4.2)
150 000IU	2.6 (2.0, 3.2)	2.9 (2.3, 3.5)	2.8 (2.2, 3.4)	3.1 (2.5, 3.7)	3.6 (3.3, 4.0)	3.5 (3.2, 3.9)	3.5 (3.1, 3.8)	3.4 (3.0, 3.7)
500 000IU	3.2 (2.6, 3.8)	3.3 (2.7, 3.9)	3.2 (2.6, 3.8)	3.1 (2.5, 3.7)	3.9 (3.6, 4.2)	3.9 (3.6, 4.2)	3.9 (3.6, 4.2)	4.0 (3.7, 4.3)

Values are arithmetic means and 95% CIs.

5.16.4 Grip Strength

Proportional between-group differences at each time point

Grip strength at each time point is shown in **figure 47** and **table 40**. There was no significant interaction between treatment group and study time point ($P = .314$), after adjustment for baseline grip strength. There was also no overall significant difference between treatment groups at any time point ($P = .826$).

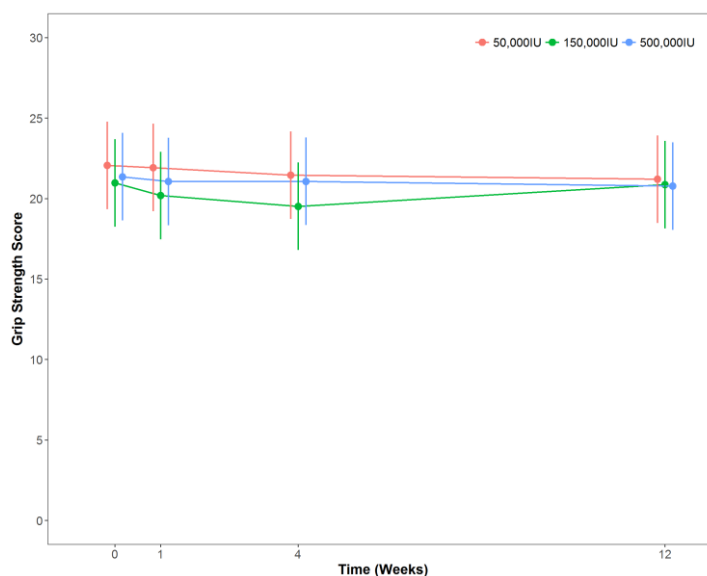
Proportional within-group changes

Grip Strength did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: -0.3 [95% CI: $-1.9, 1.3$]; difference week 4 vs. baseline: -0.3 [95% CI: $-1.9, 1.3$]; difference week 12 vs. baseline: -0.6 [95% CI: $2.2, 1.0$]), the 150 000IU treatment group (difference week 1 vs. baseline: -0.8 [95% CI: $-2.4, 0.8$]; difference week 4 vs. baseline: -1.4 [95% CI: $-3.0, 0.1$]; difference week 12 vs. baseline: 0.1 [95% CI: $-1.7, 1.5$]) and the 50 000IU treatment group (difference week 1 vs. baseline: -0.1 [95% CI: $-1.7, 1.5$]; difference week 4 vs. baseline: -0.6 [95% CI: $-2.2, 1.0$]; difference week 12 vs. baseline: -0.9 [95% CI: $-3.4, 0.7$]). Absolute values for grip strength at each time point are summarised in **table 40**.

Control group

Control group grip strength scores at baseline (mean: 19.9 [95% CI: $18.1, 21.9$]) were not significantly different to week 12 (mean: 20.3 [95% CI: $18.8, 21.8$]) ($t(26) = -0.726, P = .474$).

Figure 47: Changes in grip strength by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals (CI).

Table 40: Mean grip strength scores at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	22.1 (19.3, 24.8)	21.9 (19.2, 24.6)	21.4 (18.7, 24.2)	21.2 (18.5, 23.9)
150 000IU	21.0 (18.2, 23.7)	20.2 (17.5, 22.9)	19.5 (16.8, 22.2)	20.9 (18.1, 23.6)
500 000IU	21.4 (18.6, 24.1)	21.1 (18.3, 23.8)	21.1 (18.3, 23.8)	20.8 (18.1, 23.5)

Values are arithmetic means and 95% CIs.

5.17 Response of cardiovascular parameters to bolus dose supplementation

5.17.1 Laying to standing pulse rate ratio

Proportional between-group differences at each time point

The laying to standing pulse ratio at each time point is shown in **figure 48** and **table 41**. There was no significant interaction between treatment group and time point, after adjustment for baseline laying to standing pulse ratio ($P = .804$). There was also no overall significant difference between groups ($P = .673$).

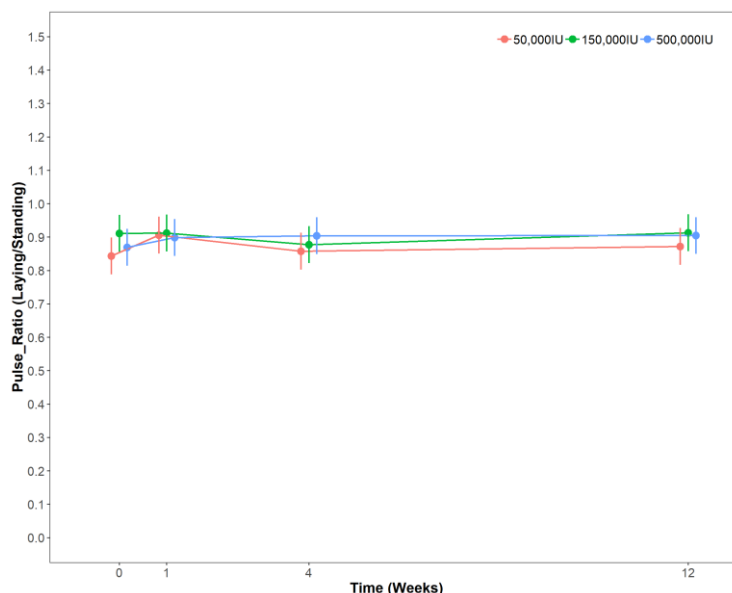
Proportional within-group changes

The laying to standing pulse ratio did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.03 [95% CI: -0.04, 0.10]; difference week 4 vs. baseline: 0.03 [95% CI: -0.03, 0.10]; difference week 12 vs. baseline: 0.04 [95% CI: -0.03, 0.10]), the 150 000IU treatment group (difference week 1 vs. baseline: -0.001 [95% CI: -0.06, 0.07]; difference week 4 vs. baseline: -0.03 [95% CI: -0.10, 0.03]; difference week 12 vs. baseline: 0.001 [95% CI: -0.06, 0.07]) and the 50 000IU treatment group (difference week 1 vs. baseline: 0.06 [95% CI: -0.004, 0.13]; difference week 4 vs. baseline: -0.01 [95% CI: -0.05, 0.08]; difference week 12 vs. baseline: 0.03 [95% CI: -0.04, 0.09]). The laying to standing pulse ratio at each time point are summarised in **table 41**.

Control group

Control group laying: standing pulse at baseline (mean: 0.87 [95% CI: 0.82, 0.92]) were not significantly different to week 12 (mean: 0.90 [95% CI: 0.86, 0.93]) ($t(26) = -1.165$, $P = .255$).

Figure 48: Changes in laying to standing pulse ratio by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 41: Mean laying to standing pulse rate at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.84 (0.79, 0.90)	0.91 (0.85, 0.96)	0.86 (0.80, 0.91)	0.87 (0.82, 0.93)
150 000IU	0.91 (0.86, 0.97)	0.91 (0.86, 0.97)	0.88 (0.82, 0.93)	0.91 (0.86, 0.97)
500 000IU	0.87 (0.81, 0.92)	0.90 (0.84, 0.95)	0.90 (0.94, 0.96)	0.90 (0.84, 0.96)

Values are arithmetic means and 95% CIs

5.18.2 Systolic blood pressure - Laying/standing ratio

Proportional between-group differences at each time point

The laying to standing diastolic blood pressure ratio at each time point is shown in **figure 49** and **table 42**.

There was no significant interaction between treatment group and time point, after adjustment for baseline laying to standing systolic blood pressure ratio ($P = .825$). There was also no overall significant difference between groups at any time point ($P = .101$).

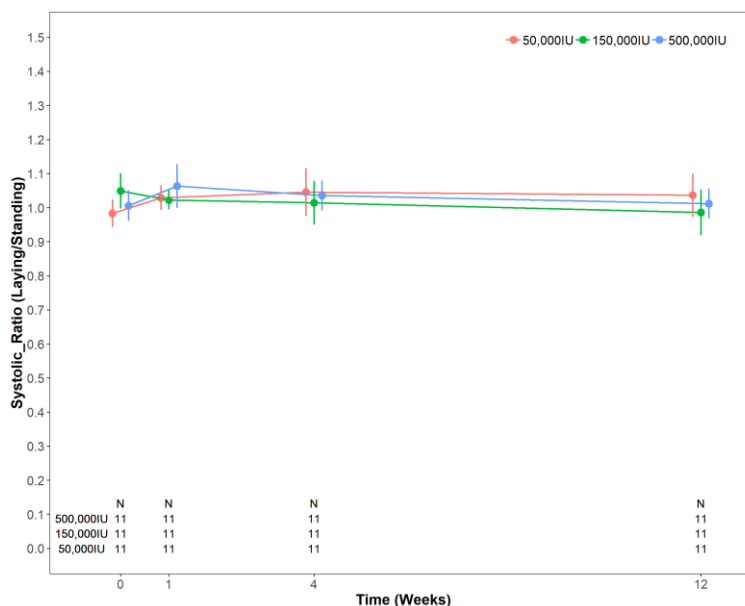
Proportional within-group changes

The laying to standing systolic blood pressure ratio did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.06 [95% CI: -0.01, 0.13]; difference week 4 vs. baseline: 0.03 [95% CI: -0.04, 0.10]; difference week 12 vs. baseline: 0.01 [95% CI: -0.06, 0.07]), the 150 000IU treatment group (difference week 1 vs. baseline: -0.03 [95% CI: -0.09, 0.04]; difference week 4 vs. baseline: -0.03 [95% CI: -0.10, 0.03]; difference week 12 vs. baseline: 0.06 [95% CI: -0.13, 0.004]) and the 50 000IU treatment group (difference week 1 vs. baseline: 0.05 [95% CI: -0.02, 0.11]; difference week 4 vs. baseline: 0.06 [95% CI: -0.01, 0.13]; difference week 12 vs. baseline: 0.05 [95% CI: -0.01, 0.12]). The laying to standing systolic blood pressure at each time point are summarised in **table 42**.

Control group

The control group laying: standing systolic blood pressure at baseline (mean: 0.99 [95% CI: 0.96, 1.03]) was not significantly different to week 12 (mean: 1.00 [95% CI: 0.97, 1.04]) ($t(26) = -.501, P = .620$).

Figure 49: Changes in laying/standing systolic blood pressure ratio by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 42: Mean laying/standing systolic blood pressure ratio at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.98 (0.93, 1.04)	1.03 (0.98, 1.08)	1.05 (0.99, 1.10)	1.04 (0.98, 1.09)
150 000IU	1.05 (1.00, 1.10)	1.02 (0.97, 1.08)	1.01 (0.96, 1.07)	0.99 (0.93, 1.04)
500 000IU	1.01 (0.95, 1.06)	1.06 (1.01, 1.12)	1.04 (0.98, 1.09)	1.01 (0.96, 1.07)

Values are arithmetic means and 95% CIs.

5.19.3 Diastolic blood pressure - Laying: standing ratio

Proportional between-group differences at each time point

The laying: standing systolic blood pressure at each time point is shown in **figure 50** and **table 43**. There was no significant interaction between treatment group and time point, after adjustment for baseline laying/standing systolic blood pressure ratio ($P = .331$). There was also no overall significant difference between groups ($P = .886$).

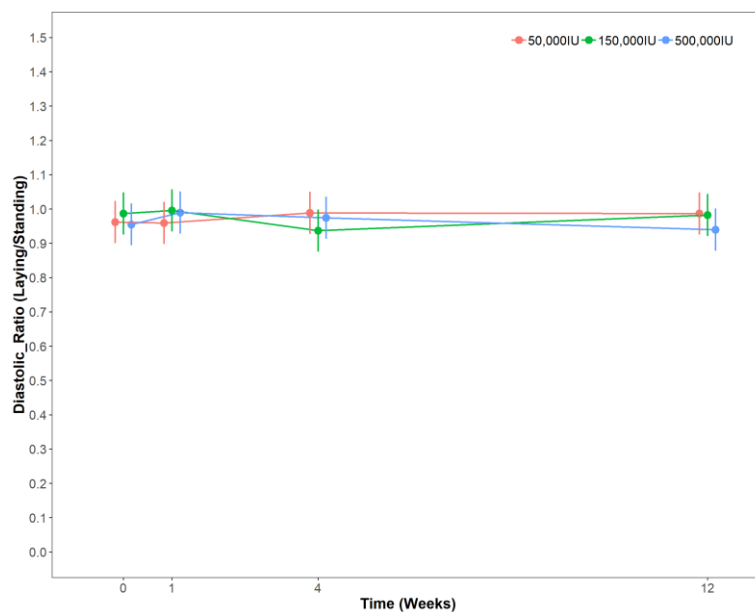
Proportional within-group changes

The laying to standing diastolic blood pressure ratio did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: 0.03 [95% CI: -0.04, 0.11]; difference week 4 vs. baseline: 0.02 [95% CI: -0.05, 0.09]; difference week 12 vs. baseline: -0.02 [95% CI: -0.09, 0.06]), the 150 000IU treatment group (difference week 1 vs. baseline: 0.01 [95% CI: -0.06, 0.08]; difference week 4 vs. baseline: -0.05 [95% CI: -0.12, 0.02]; difference week 12 vs. baseline: 0.004 [95% CI: -0.08, 0.07]) and the 50 000IU treatment group (difference week 1 vs. baseline: -0.003 [95% CI: -0.08, 0.07]; difference week 4 vs. baseline: 0.03 [95% CI: -0.05, 0.10]; difference week 12 vs. baseline: 0.02 [95% CI: -0.05, 0.10]). The laying: systolic diastolic blood pressure at each time point is summarised in **table 43**.

Control group

The control group laying: standing diastolic blood pressure at baseline (mean: 0.97 [95% CI: 0.91, 1.03]) was not significantly different to week 12 (mean: 0.95 [95% CI: 0.93, 0.98]) ($t(26) = -0.501$, $P = .683$).

Figure 50: Changes in laying/standing diastolic blood pressure ratio by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 43: Mean laying/standing diastolic blood pressure ratio at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	0.96 (0.90, 1.02)	0.96 (0.90, 1.02)	0.99 (0.93, 1.05)	0.99 (0.93, 1.05)
150 000IU	0.99 (0.93, 1.05)	1.00 (0.93, 1.06)	0.94 (0.88, 1.00)	0.98 (0.92, 1.04)
500 000IU	0.95 (0.89, 1.02)	0.99 (0.93, 1.05)	0.97 (0.91, 1.04)	0.94 (0.88, 1.00)

Values are arithmetic means and 95% CIs.

5.20.4 Aldosterone-Renin Ratio (ARR)

Proportional between-group differences at each time point

The ARR at each time point is shown in **figure 51** and **table 44**. There was no significant interaction between treatment group and time point ($P = .567$), after adjustment for baseline ARR. There was also no overall significant difference between groups ($P = .861$) in the ARR at any time point.

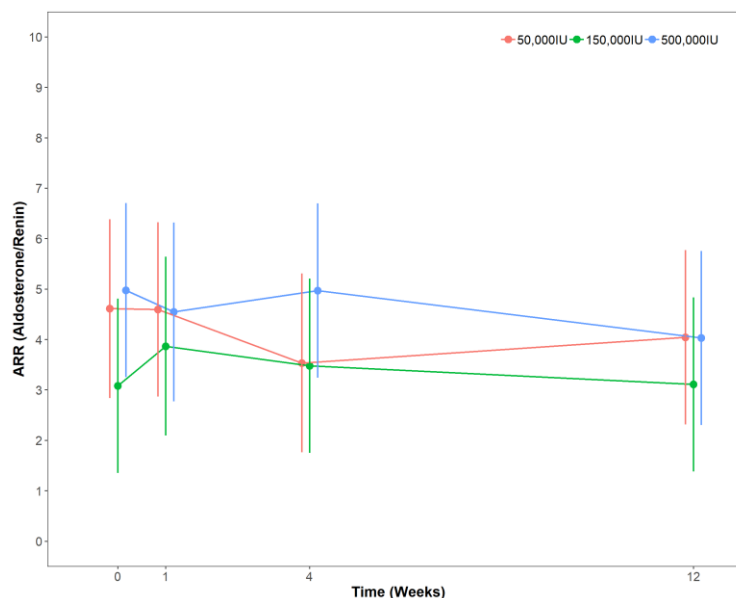
Proportional within-group changes

The ARR did not change from baseline levels at any time point in the 500 000IU treatment group (difference week 1 vs. baseline: -0.43 [95% CI: -1.99, 1.12]; difference week 4 vs. baseline: -0.01 [95% CI: -1.51, 1.49]; difference week 12 vs. baseline: -0.94 [95% CI: -2.45, 0.55]), the 150 000IU treatment group (difference week 1 vs. baseline: 0.78 [95% CI: -0.77, 2.34]; difference week 4 vs. baseline: -0.40 [95% CI: -1.11, 1.90]; difference week 12 vs. baseline: 0.03 [95% CI: -1.48, 1.53]) and the 50 000IU treatment group (difference week 1 vs. baseline: -0.02 [95% CI: -1.57, 1.53]; difference week 4 vs. baseline: -1.08 [95% CI: -2.68, 0.52]; difference week 12 vs. baseline: -0.57 [95% CI: -2.12, 0.98]). The ARR at each time point is summarised in **table 44**.

Control group

The control group ARR at baseline (mean: 3.5 [95% CI: 2.7, 4.3]) was not significantly different to week 12 (mean: 3.5 [95% CI: 2.2, 4.7]) ($t(26) = .113$, $P = .911$).

Figure 51: Changes in ARR (ng/dL per ng/ml/h) by treatment group.



Red lines represent the 50 000IU dose, green lines represent the 150 000IU dose and blue lines represent the 500 000IU dose. Values are arithmetic means and bars represent 95% Confidence Intervals.

Table 44: Mean ARR (ng/dL per ng/ml/h) at each time point.

Treatment Group	Visit (Weeks)			
	0	1	4	12
50 000IU	4.61 (2.84, 6.38)	4.59 (2.87, 6.32)	3.53 (1.76, 5.31)	4.04 (2.32, 5.77)
150 000IU	3.08 (1.35, 4.81)	3.87 (2.09, 5.64)	3.48 (1.75, 5.21)	3.11 (1.38, 4.83)
500 000IU	4.98 (3.25, 6.70)	4.54 (2.77, 6.32)	4.97 (3.24, 6.69)	4.03 (2.30, 5.75)

Values are arithmetic means and 95% CIs.

Discussion

5.21 PTH

PTH did fall in each treatment group, but there was no clear dose-response fall. PTH had fallen significantly by week 4 in the largest dose group but had returned to baseline levels by week 12. PTH fell significantly from baseline by week 1 in the 150 000IU group and was significantly lower than baseline at week 12. PTH in the lower dose group was only significantly lower than baseline at week 12.

Most other studies have shown a significant decrease in PTH after a large single bolus of vitamin D₃ which occurs as early as 3-days after administration (172, 214, 219, 313) and 1-week after administration (311), especially at doses greater than 100 000IU. These decreases in PTH have also been shown to be sustained for period of up to 30-days (173, 312), 60-days (172), 90-days (212, 214, 219, 313), 180-days (215-217) and 270-days (201) after administration.

However, results across studies are heterogeneous and there is also evidence from bolus dosing studies that a decrease in PTH does not always occur and that any decrease may not always be sustained over the reported study period. Bacon et al only report decreases in PTH after a 500 000IU bolus in those with low 25(OH)D and in those with sufficient 25(OH)D PTH was unaffected across the study period of 9-months (213). Some studies, using smaller bolus doses of vitamin D₃, do not demonstrate decreases in PTH at all (200, 218, 245).

There are a wide range of other factors that might explain why PTH may not always respond to vitamin D supplementation. Dietary calcium intake has been shown to influence PTH response to vitamin D supplementation. In this study, calcium intake was lower than the RNI of 700mg/day in the 50 000IU (677mg/day) and 150 000IU (637mg/day) treatment groups. A higher treatment effect has previously been observed with combined calcium and vitamin D treatment compared to vitamin D monotherapy. The low baseline calcium intake in the lower dose groups may therefore attenuate the PTH response to supplementation (180) in this study. However, this does not explain the lack of a clear and sustained effect of supplementation on PTH in the largest dose group where dietary calcium was well above the RNI (1013mg/day).

It has also been reported that the treatment effect of vitamin D on PTH is less in people aged >50 years compared to those who are aged <50 years (180). This may explain why we do not see a clear and sustained decrease in PTH in this study. PTH response may be attenuated in older age, because the 25(OH)-PTH axis may be disturbed with increasing age. With increasing age for any given concentration of 25(OH)D, PTH is higher (178, 185) and this may be explained by poorer renal function (185) and intestinal 1, 25(OH)₂D resistance in older adults (186, 187). Mean baseline PTH was also well within the normal range, indicating that despite vitamin D deficiency, participants in this study did not have secondary hyperparathyroidism. PTH response may be attenuated in participants where PTH is not elevated at baseline.

5.22 Serum calcium and Ionised calcium

Despite the large increases in vitamin D metabolites (reported in chapter 4), including total 25(OH)D, 1,25(OH)₂D, free 25(OH)D and free 1, 25(OH)₂D, there was no treatment effect on sCa or iCa. SCa and iCa did not change across the study period in any of the treatment groups.

This demonstrates that despite the large amount of vitamin D₃ entering circulation as a one-off dose, the homeostatic mechanisms that maintain sCa and iCa within the normal range remain extremely robust. These findings do not support the hypothesis that the increases in falls and fracture after a large bolus dose of vitamin D₃ are caused by disproportionate rises in free vitamin D metabolites that lead to hypercalcemia.

Other bolus dosing studies also confirm that the homeostatic mechanism that keeps serum calcium in the normal range is very robust. Almost all other bolus dosing studies that have reported sCa or iCa show no change from baseline values (200, 216, 218, 245, 286, 311-313) and no hypercalcemia (201, 215, 245). Only one study has reported a significant rise in serum calcium from baseline (+0.025mmol/l [SD: +/- 0.075]) (214), three-days after a single large bolus dose (600 000IU). However, mean values remained well within the normal range and quickly fell to baseline levels. There are some sporadic reports of hypercalcemia in some bolus dosing studies (200, 212, 216, 311). However, in some cases these may be explained by mild primary hyperparathyroidism (200).

5.23 Urinary calcium excretion

The urCa:urCr increased from baseline at 1-week and remained elevated at 4-weeks post-administration in the largest dose groups.

This increase in urinary calcium excretion is in line with findings from the limited number of other vitamin D₃ bolus dosing studies that have reported urinary calcium excretion. In these studies, increases in urinary calcium excretion have been reported across a heterogeneous range of D₃ bolus doses and populations (200, 214, 217, 311).

Overall, findings from this study and other reported findings in the literature reaffirm that the homeostatic mechanism that keeps circulating calcium levels in the normal range is an extremely robust one. In the distal tubules of the kidney, calcium reabsorption is regulated by PTH and 1, 25(OH)₂D via a transcellular mechanism (67). Any excess in circulating calcium after a bolus dose of vitamin D₃ (due to sharp increases in free 25(OH)D and free 1, 25(OH)₂D as shown in chapter 4) appears to be readily filtered out through the distal tubules and lost in urine. This keeps sCa and iCa within the normal ranges. We saw that PTH significantly fell in each treatment group at various time points and it is likely that the slight fall in PTH helps to sustain the loss in calcium through the distal tubules, despite the reported increased in 1, 25(OH)₂D and other vitamin D metabolites.

5.24 Serum Phosphate and FGF-23

There was no observed effect of vitamin D supplementation on serum phosphate. Despite this, FGF-23 increased by 25% from baseline at week-1 in the largest dose group but returned to baseline levels by week-4. There was also a 26% increase in FGF-23 by week-4 in the lowest dose group. Therefore, despite little change in serum phosphate levels across the study period, there was some evidence of an increase in FGF-23.

Surprisingly, few studies have reported changes in serum phosphate after a single bolus dose of vitamin D₃. Cipriani et al reported an immediate increase in serum phosphate from baseline levels after just 3-days post-administration of a 600 000IU D₃ oral bolus, but levels had fallen to reported at baseline by 15-days follow-up (214). Studies with longer durations between baseline and follow-up have not reported increases in serum phosphate (216, 217), indicating that phosphate levels may quickly return to baseline levels after a vitamin D₃ oral bolus. It is possible that an immediate increase in serum phosphate may have occurred before our first follow-up time point at 1-week post-administration.

FGF-23 appears to downregulate the synthesis of 1, 25(OH)₂D by inhibiting the transcription of CYP27B1 at the kidney (59) and increases the action of the 24-hydroxylase enzymes that catalyse the degradation of 25(OH)D and 1, 25(OH)₂D. The increase in FGF-23 demonstrated in the two of the treatment groups, coupled with the fall in PTH observed by various time points in all treatment groups, suggests that the catabolic pathways for vitamin D metabolites respond rapidly to the sharp increases in vitamin D metabolites in circulation (shown in chapter 4) after a large bolus dose. Taken together this again reinforces the theory that the homeostatic mechanisms for the vitamin D and calcium are robust.

5.25 VDBP and albumin

Findings from this study demonstrate that bolus dose vitamin D₃ supplementation did not have any effect on the levels of carrier proteins for vitamin D metabolites. This is in line with findings from the only other study that has measured albumin and VDBP after a vitamin D₃ oral bolus (245).

5.26 Bone turnover markers

We did not see a dose-response change in any of the measured bone turnover markers. However, there was some evidence of a within-group transient increase in each of the markers at week 1 in the 500 000IU treatment group. Osteocalcin increased by approximately 23%, PINP by 9% and CTX by 26%. Osteocalcin and CTX had fallen to baseline levels by week 4, but PINP remained elevated at week 4 by approximately 15%, before falling to baseline levels by week 12. Bone turnover markers were similar to baseline levels at all time points in the lower dose treatment groups.

This transient increase in bone resorption markers after a very large single large bolus dose has been demonstrated before. After a 600 000IU bolus, increases in CTX from baseline of between 23-35% were

observed up to 60-days post-administration (219). There was also a smaller, but significant, transient increase in CTX 3-days after administration of a smaller bolus dose of 300 000IU (219).

Our findings that bone turnover markers did not change over time in the 150 000IU and 50 000IU treatment groups are consistent with findings from other studies. After bolus doses of 200 000IU and 100 000IU, there was no significant changes in CTX from baseline at 1-week and 3-month post administration in healthy older women (311). Other bolus dosing studies have only measured bone turnover markers after at least 1-month post-supplementation. These studies also appear to show either no change or a decrease in bone formation markers (200, 213, 217).

Higher levels of bone turnover is associated with bone loss and some studies have shown that higher markers of bone turnover are associated with a greater risk of fracture (319). It is not clear if these transient changes in increases in BTMs immediately after bolus dosing that have been found in this study, and by Rossini et al (219), are clinically relevant, but this mechanism may help to explain the increase in fractures immediately after administration that has previously been reported and requires further investigation.

5.27 Physical function

Overall data from this study indicates that bolus dose vitamin D₃ does not appear to have adverse effects of physical function. As previously described, some studies have demonstrated a link between single large bolus doses of vitamin D (8, 9) and large repeated dose of vitamin D₃ (10) and an increase in falls and fracture. Our data would not support a decline in physical function as the explanation for these findings.

We did not observe any between-group differences in overall SPPB scores. Overall SPPB scores were not different to baseline at any time point in the largest dose groups. There was a very small increase in repeated chair stand score in the 50 000IU treatment group from baseline at week 1 that was maintained at week 12.

We did not see a benefit of supplementation in the largest bolus dose groups, but in the context of this study, it is important to note that we did not see any adverse effects on physical function measures in these treatment groups. We did comfortably reach and even exceed levels of total 25(OH)D that was reported in previous studies that have demonstrated adverse events after bolus dose vitamin D. There are contrasting findings from the limited number of studies that have investigated the effect of a single large oral bolus of vitamin D₃ on physical performance. One study reports significant increases in SPPB scores 12-weeks after 600 000IU, although the absolute increase in SPPB score was extremely small (212). The same study also reported a significant increase in quadriceps muscle strength (212), but again, this increase was small and it is not clear if these small increases are clinically relevant. In contrast, physical function assessments of participants from the Sanders et al (8) study showed that there was a greater decline in muscle strength in those who demonstrated the greatest fluctuation in total 25(OH)D levels from baseline.

The small increase in repeated chair stand score in the lowest dose group is interesting. It may simply be a random finding. However, the repeated chair stand is a measure of lower limb strength, which is particularly important in the context of falls as lower extremity function is a major risk factor for falls and fracture (320) and proximal muscle weakness is a symptom of vitamin D deficiency (321). There is evidence from other studies that lower dose vitamin D supplementation may improve physical function and muscle strength and reduce falls (115-118). Although, these findings are inconsistent with other studies showing no effect on physical function, muscle strength and falls (318). Findings from studies are difficult to interpret and overall conclusions are hard to make due to the heterogeneity in populations studied, dosing regimen and outcomes measures used to assess physical function.

There were also no between-group differences in grip strength and grip strength scores did not differ from baseline at any time point in any treatment group. Other D₃ oral bolus dosing studies have not shown any effect on grip strength (313). Although grip strength did increase in one study after a 500 000IU D₃ bolus given to older hospitalized patients, it did so in other vitamin D treatment groups as well and there was no placebo group for comparison (213). Grip strength is not always lower in people with vitamin D deficiency and several systematic reviews have shown that grip strength is not always improved with vitamin D supplementation in older adults, although there is wide heterogeneity between studies (118, 322).

Overall, findings in this study do not support a link between vitamin D and adverse physical function, and therefore an adverse effect of bolus dose vitamin D does not appear to explain previous findings of an increase in falls and fracture after large doses of vitamin D.

However, these findings should be treated with caution. Although vitamin D deficient at baseline, the women in this study were otherwise healthy and were certainly not considered frail. The populations in studies where falls and fracture have been previously reported after large doses of vitamin D were much older and potentially frailer cohorts, with at least one prior fall (10) or considered at high risk of fracture (8). We must consider the possibility that any adverse effects on physical function of large doses of vitamin D may only present in weaker population sub-groups.

It is also possible that, because this was a healthy older population, the SPPB that was used in this study to assess physical function may not have been sensitive enough to detect any adverse effects. More subtle effects on balance and co-ordination may not have been detected using this method. Further research may investigate potential subtle effects of bolus dose vitamin D on motor function, gait, co-ordination and balance using wearable movement sensors (e.g. APDM/OPAL sensors) that may not have been picked up by the SPPB.

5.28 Cardiovascular outcomes

The data reported in this study do not demonstrate an effect of a bolus dose vitamin D on the assessed cardiovascular outcomes. Despite the large dose-response effect of treatment on 25(OH)D and other vitamin D metabolites, we did not observe any corresponding treatment effect on the laying/standing pulse ratio and laying/standing systolic blood pressure ratio or the laying/standing-diastolic blood pressure ratio. There was

also no treatment effect on the ARR. This is the first study to report the effects of a single large D₃ bolus on the ARR.

Previous research has demonstrated a blood pressure lowering effect of vitamin D that is thought to be mediated through the renin-angiotensin system (238-240). Clinical studies have also demonstrated a fall in systolic and diastolic blood pressure after vitamin D supplementation (124) . It has been hypothesised that a single large bolus dose of vitamin D may lead to postural hypotension and that this may be the mechanism of the increased rate of falls reported in some studies. However, our data do not support this hypothesis.

Only one previous study has investigated the effects of a single large bolus dose of vitamin D₃ on blood pressure, which also demonstrated no significant effect of a 200 000IU D₃ oral bolus on blood pressure. Our data is consistent with these findings and also demonstrate that no effect on blood pressure is observed after an even larger bolus.

Caution is again required in the interpretation these findings. The ARR is affected by a wide variety of factors and as a result is a noisy variable (323). A larger sample size may be required to give the study adequate power to detect any treatment effect on the ARR.

Some protocols suggest that to improve sensitivity and reduce measurement variability a liberal salt diet should be encouraged and the collection of blood midmorning from seated patients following 2-4 h upright posture improves sensitivity (323). The practicalities of the study did not allow for these procedures to be incorporated into the protocol. Despite this, we saw no evidence from the data that ARR was at all affected by vitamin D treatment.

Chapter conclusions

Despite a large dose-response increase in vitamin D metabolites (chapter 4) after a vitamin D₃ bolus, there was no effect on sCa and iCa and no evidence of hypercalcemia in any treatment group. Therefore, hypercalcemia is unlikely to explain the increase in falls and fracture after a large bolus dose of vitamin D that has previously been reported. There were also no adverse effects on physical function, assessed by a SPPB and grip strength, in any of the treatment groups and there was no evidence of any treatment effect on blood pressure or ARR. Therefore, it also appears to be unlikely that direct adverse effects of vitamin D metabolites on muscle or postural hypotension are explanations for the increase in falls after large bolus doses of vitamin D that has been previously established.

In this chapter we also see evidence that the homeostatic mechanisms for the vitamin D and calcium systems are extremely robust and that single large bolus doses of vitamin D₃ up to 500 000IU are tolerable and safe, certainly in a vitamin D deficient, but otherwise healthy post-menopausal population. This is evidenced by the significant within-group falls in PTH after supplementation and the significant increases in FGF-23, particularly in the largest dose group. There were also corresponding increases in urinary calcium excretion in the highest dose groups. Taken together, these findings suggest that the catabolic pathways are effective at maintaining levels of serum calcium within the normal range, after a bolus dose of vitamin D₃ of up to 500 000IU.

An interesting finding was the confirmation of a transient increase in bone turnover markers at 1-week in the 500 000IU treatment group. Similar transient increases were not observed in the lower dose groups. Whether this increase in bone turnover immediately after a large bolus dose is clinically significant and whether this could explain the increase in fracture after a single large bolus dose requires further investigation.

Findings in this chapter should be extrapolated with caution. The study population was an older group who were vitamin D deficient at baseline, but otherwise healthy. This is in contrast to other studies that have reported an increase in falls and fracture after large doses of vitamin D. In those studies participants have tended to be older and frailer than the population studied here. We must consider the possibility that any adverse effects of large doses of vitamin D may only present in weaker population sub-groups.

Chapter 6:

Overall Summary &

Conclusions

Background

Several studies had reported adverse events, including an increase in falls and fracture, after a single bolus dose of vitamin D (8, 9), but the mechanism for any increase in falls and fracture has not yet been explained.

One proposed mechanism was that the binding capacity of VDBP can become saturated in some situations (such as after a single large bolus dose). Therefore, the increased rate of falls and fractures seen in these studies may have been due to vitamin D toxicity in the initial period after administration of a large bolus dose due to the binding capacity of VDBP being overwhelmed by the large influx of vitamin D into circulation (231). It was hypothesised that this may lead to a sharp increase in total 25(OH)D with a relatively greater increase in free 25(OH)D. In addition, there may also be a disproportionate rise in free 1, 25(OH)₂D, because this metabolite has the weakest binding affinity for VDBP. There may then be several consequences of disproportionate increases in one or more of these free vitamin D metabolites, such as hypercalcemia, that may increase the propensity for people to fall (231). Vitamin D is a potent suppressor of renin synthesis (238, 239, 240) and excess free metabolites could negatively impact upon the renin-angiotensin system, leading to postural hypotension and possibly leading to falls. Elevated free metabolites may also have direct effects on the brain (the VDR is intensely expressed in the cerebellum which is heavily involved in balanced muscular activity) (101) and/or direct effects on muscle, although it is controversial whether the VDR is expressed in human muscle tissue (110, 112, 235).

No bolus dosing study had previously set out to mechanistically understand the mechanism for the increase in falls and fracture that had been demonstrated in some vitamin D bolus dosing studies. Therefore, this study set out to investigate the clinically important hypothesis that a disproportionate rise in free vitamin D metabolites and hypercalcemia could explain the increase in falls and fractures after high dose vitamin D that have been previously reported.

Methods

The study was a single centre, double-blinded, randomised, controlled trial to determine the effects of three different oral bolus doses of vitamin D₃ (50 000IU, 150 000IU and 500 000IU) on total and free 25(OH)D and total 1, 25(OH)₂D and free 1, 25(OH)₂D in vitamin D deficient, but otherwise healthy, postmenopausal women. Thirty-three vitamin D deficient (25(OH)D <30nmol/l) postmenopausal women were randomized to one of the three treatment groups. Twenty-seven vitamin D sufficient (25(OH)D >50nmol/l) postmenopausal women were recruited as a concurrent control group. Treatment participants attended four study visits (after screening) at baseline and 5(+/-2), 28(+/-3), and 84(+/-5) days after administration of the vitamin D bolus. A range of biochemical measurements were made, including total 25(OH)D, 25(OH)D₃ (measured by LC-MS/MS), free

25(OH)D (directly measured and calculated), PTH, serum calcium, ionized calcium and urinary calcium and bone turnover markers. Grip strength and a SPPB were used to assess muscle strength and function. Cardiovascular outcomes were also assessed, including postural changes in blood pressure and the ARR.

Overview of key findings

Changes in total and free vitamin D metabolites (chapter 4)

Despite a large dose-response effect of supplementation on total 25(OH)D and total 1, 25(OH)₂D, there was no evidence of a disproportionate rise in free metabolites. The proportional increases in free 25(OH)₂D (either calculated or directly measured) were in line with the proportional increases in total 25(OH)D (and 25(OH)D₃) across all study time points in all treatment groups. Similarly, the proportional increases in free 1, 25(OH)₂D were in line with the proportional increases in total 1, 25(OH)₂D across all time points in all treatment groups. The proportional increases for total and vitamin D metabolites at week 1 (where any disproportionate rise in free metabolites after a large bolus dose appears most likely to occur) in each treatment group are summarised in **table 45**.

The percentage free 25(OH)D (derived from the ratios of calculated free 25(OH)D to total 25(OH)D and calculated free 25(OH)D₃ to total 25(OH)D₃) were not different between treatment groups at any time point and do not change across the study period. The percentage free that was derived from the ratio of measured free 25(OH)D to total 25(OH)D show a slight increase from baseline at week 1 in the 500 000IU treatment group that was significantly different compared to the other treatment groups. However, the percent free remained in the normal range and was comparable with percentages reported in healthy adults (0.02-0.09%) (156, 165). The percent free 1, 25(OH)₂D did not change from baseline in any treatment group and did not differ between treatment groups at any time point. The percent free 1, 25(OH)₂D in all treatment groups and at all time points was also in line with the 0.4% reported by other authors in healthy participants (156).

Taken together, the data presented indicates that there is little evidence to support the hypothesis of a disproportionate rise in free 25(OH)D or free 1, 25(OH)₂D after a single large bolus dose in this vitamin D deficient, but otherwise healthy, older population. It is therefore, unlikely that the adverse events reported by Sanders et al (8) after a 500 000IU oral bolus, and in other studies that have reported similar adverse events to large doses of vitamin D, are caused by excess or disproportionate levels of free vitamin D metabolites.

Table 45: A summary of the proportional increases in vitamin D metabolites from baseline at week 1 in each treatment group.

Vitamin D metabolite	Treatment group	Proportional increase from baseline at week 1
Total 25(OH)D	500 000IU	4.5
	150 000IU	2.2
	50 000IU	1.6
Total 25(OH)D ₃	500 000IU	7.2
	150 000IU	3.4
	50 000IU	2.3
Measured free 25(OH)D	500 000IU	7.1
	150 000IU	2.1
	50 000IU	1.7
Calculated free 25(OH)D	500 000IU	4.3
	150 000IU	2.3
	50 000IU	1.6
Calculated free 25(OH)D ₃	500 000IU	6.9
	150 000IU	3.5
	50 000IU	2.3
Total 1, 25(OH) ₂ D	500 000IU	2.3
	150 000IU	1.8
	50 000IU	1.3
Calculated free 1, 25(OH) ₂ D	500 000IU	2.2
	150 000IU	1.8
	50 000IU	1.3

Changes in other variables (chapter 5)

Despite the large increases in vitamin D metabolites, there was no evidence of hypercalcemia in any treatment group. There was no treatment effect on sCa or iCa and sCa and iCa did not change across the study period within any of the treatment groups. This demonstrates that despite the large amount of vitamin D₃ entering circulation as a one-off dose, the homeostatic mechanisms that maintain sCa and iCa within the normal range remain extremely robust. These findings do not support the hypothesis that the increases in falls and fracture after a large bolus dose of vitamin D₃ are caused by disproportionate rises in free vitamin D metabolites that lead to hypercalcemia.

There was evidence of a fall in PTH in all treatment groups (although there was no dose-response effect) and there was some evidence of increases in FGF-23 and increases in urinary calcium excretion after bolus dosing. The increase in FGF-23 and urinary calcium excretion demonstrated in the two of the treatment groups, coupled with the fall in PTH observed at different time points in all treatment groups, suggests that the catabolic pathways for vitamin D metabolites respond rapidly to the sharp increases in vitamin D metabolites in circulation after a large bolus dose. Taken together this again reinforces the theory that the homeostatic mechanisms for the vitamin D and calcium are robust and effectively prevent hypercalcemia after a single large oral dose of vitamin D₃ in healthy older adults.

There was also no effect of treatment on cardiovascular outcomes, including blood pressure and ARR. Despite the large dose-response effect of treatment on 25(OH)D and other vitamin D metabolites, we did not observe any corresponding treatment effect on the laying/standing pulse ratio and laying/standing systolic blood pressure ratio or the laying/standing-diastolic blood pressure ratio. Clinical studies have also demonstrated a fall in systolic and diastolic blood pressure after vitamin D supplementation (124). It has been hypothesised that a single large bolus dose of vitamin D may lead to postural hypotension and that this may be the mechanism of the increased rate of falls reported in some studies. However, our data do not support this hypothesis.

Vitamin D receptors may be present in muscle (110). Increased concentrations of free vitamin D metabolites interacting with the VDR in muscle cells and influencing the activity of the cells has been postulated as an explanation in the increase in falls after a single large bolus of D₃. However, there were no adverse effects of treatment after a single large bolus dose on physical function, assessed by SPPB and grip strength. There was a small improvement in overall SPPB scores on the lowest dose group that was not demonstrated with the higher doses, although the absolute increase was small and whether this increase is clinically relevant is not clear. Overall data from this study indicates that bolus dose vitamin D₃ does not appear to have adverse effects of physical function. We did not see a benefit of supplementation in the largest bolus dose groups, but in the context of this study, it is important to note that we did not see any adverse effects on physical function measures in these treatment groups. As previously described, some studies have demonstrated a link between single large bolus doses of vitamin D (8, 9) and large repeated dose of vitamin D₃ (10) and an increase in falls and fracture. Our data would not support a decline in physical function as the explanation for these findings.

Interestingly, there was evidence of a transient increase in bone turnover markers 1-week after administration in the 500 000IU treatment group. Osteocalcin increased by approximately 23%, PINP by 9% and CTX by 26%. Osteocalcin and CTX had fallen to baseline levels by week 4, but PINP remained elevated at week 4 by approximately 15%, before falling to baseline levels by week 12. The transient increase in CTX, OC and PINP was

not observed with the lower doses. This is in line with previous findings that have also demonstrated a transient increase in bone resorption markers after a large bolus dose (219). Higher levels of bone turnover is associated with bone loss and some studies have shown that higher markers of bone turnover are associated with a greater risk of fracture (319). It is not clear if these transient changes in increases in BTMs immediately after bolus dosing that have been found in this study, and by Rossini et al (219), are clinically relevant, but this mechanism may help to explain the increase in fractures immediately after administration that has previously been reported and requires further investigation.

Table 46 gives a summary of the study hypothesis that have been accepted and rejected based on the data collected from the study. **Table 47** gives a simple summary of changes from baseline of vitamin D metabolites by treatment group and **table 48** provides a simple summary of changes from baseline in all other variables by treatment group.

Summary

To conclude, a single large bolus is up to 500 000IU appears to be well tolerated in healthy older adults. There was little evidence of a disproportionate rise in free vitamin D metabolites after a single large bolus dose (up to 500 000IU). The data also does not suggest that hypercalcemia, poorer physical function and postural hypotension explain the increase in falls that had been previously reported after a large bolus. The exact mechanism of falls remains unclear. A transient increase in BTMs 1-week after a 500 000IU bolus dose may explain the increase in fractures immediately after large bolus dose administration that has previously been reported and requires further investigation.

Table 46: A summary of the acceptance or rejection of study hypotheses. Green indicates strong evidence for accepting the hypothesis; orange indicates not enough evidence to fully accept the hypothesis and red indicates strong evidence to reject the hypothesis.

Experimental Hypotheses	Accepted or rejected	Data to support conclusion
1. There will be a dose-response rise in total 25(OH)D and total 1,25(OH) ₂ D after bolus dosing of vitamin D ₃ by week 1	Accepted	There was a clear dose-response increase in total metabolites at week 1 (chapter 4)
2. There will be a dose-response rise in free 25(OH)D and free 1,25(OH) ₂ D after bolus dose supplementation of vitamin D ₃ by week 1	Accepted	There was a clear dose-response increase in free metabolites at week 1 (chapter 4)
3. There will be a disproportionate rise in free 25(OH)D in response to the largest dose of vitamin D ₃ (500 000IU) 1 week after administration	Rejected	There was little evidence of a disproportionate rise in free 25(OH)D. The dose response increases in free 25(OH)D at week 1 (and proportional differences from baseline at other timepoints) were of similar magnitude to the increases reported for total 25(OH)D in all treatment groups. All measures of free 25(OH)D remained in the normal range and the percent free in all treatment groups at all time points was comparable with percentages reported in healthy adults (chapter 4)
4. There will be a disproportionate rise in free 1, 25(OH) ₂ D in response to the larger bolus dose of vitamin D ₃ (500 000IU) 1 week after administration	Rejected	There was no evidence of a disproportionate rise in free 1, 25(OH) ₂ D. The dose response increases in free 1, 25(OH) ₂ D at week 1 (and proportional differences from baseline at other timepoints) were similar in magnitude to the increase reported for total 1, 25(OH) ₂ D in all treatment groups. The percentage free 1, 25(OH) ₂ D did not change within any treatment group and did not differ between treatment groups at any time point. The percentage free reported in all treatment groups was in line with that reported in healthy participants (chapter 4)
5. There will be a dose-response rise in albumin and vitamin D binding protein after bolus dose supplementation of vitamin D ₃ 1-week after administration	Rejected	There were no between-group differences or within group changes over time in carrier proteins. Therefore, bolus dose supplementation had no effect on carrier protein levels (chapter 5)
6. There will be a dose-response decrease in PTH in response to bolus dose supplementation	Rejected	PTH did fall in each treatment group but there was no clear dose-response fall (chapter 5)
7. There will be an increase in serum calcium and serum ionised calcium in the largest bolus dose group at 1-week after administration	Rejected	There was no treatment effect on sCa and iCa. sCa and iCa did not change across the study period in any of the treatment groups (chapter 5)
8. There will be a dose-response increase in 24-hour urCa:urCr in response to bolus dose supplementation	Rejected	urCa:urCr did increase significantly from baseline by week 1 in the largest dose groups but did not change from baseline at any time point in the lowest dose group. There was no clear dose-response increase (chapter 5).
9. There will be a dose-response increase in PINP, OC and CTX in response to bolus dose supplementation	Rejected	There was a transient increase in all measured BTMs by week 1 in the 500 000IU group only (chapter 5).
10. There will be an improvement in SPPB and grip strength scores in the lower dose groups, but the higher dose group will have a negative effect on SPPB and grip strength scores	Rejected	There was no effect of treatment on overall SPPB scores or grip strength scores (chapter 5).
11. There will be a dose-response decrease in systolic and diastolic blood pressure and ARR in response to bolus dose supplementation	Rejected	There was no evidence of any effect of treatment on laying:standing systolic or diastolic blood pressure and no evidence of any effect on the ARR (chapter 5).

Table 47: A summary of within-group changes from baseline to vitamin D metabolites by treatment group. Up-arrows (↑) indicate a significant increase from baseline. Down-arrows (↓) indicate a significant decrease from baseline values. A ↔ indicates no difference compared to baseline values.

Variable	Treatment group (IU)	Change at week 1 vs. baseline	Change at week 4 vs. baseline	Change at week 12 vs. baseline
Total 25(OH)D	500 000	↑	↑	↑
(immunoassay)	150 000	↑	↑	↑
	50 000	↑	↑	↑
Total 25(OH)D ₃	500 000	↑	↑	↑
(LC-MS/MS)	150 000	↑	↑	↑
	50 000	↑	↑	↑
Total 1, 25(OH) ₂ D	500 000	↑	↑	↑
	150 000	↑	↑	↑
	50 000	↑	↑	↔
Measured free 25(OH)D	500 000	↑	↑	↑
	150 000	↑	↑	↑
	50 000	↑	↑	↑
Calculated free 25(OH)D (immunoassay)	500 000	↑	↑	↑
	150 000	↑	↑	↑
	50 000	↑	↑	↑
Calculated free 25(OH)D ₃ (LC-MS/MS)	500 000	↑	↑	↑
	150 000	↑	↑	↑
	50 000	↑	↑	↑
Calculated free 1, 25(OH) ₂ D	500 000	↑	↑	↑
	150 000	↑	↑	↑
	50 000	↑	↑	↑
Percent measured free 25(OH)D	500 000	↑	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Percent calculated free 25(OH)D	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Percent calculated free 1, 25(OH) ₂ D	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔

Table 48: A summary of within-group changes from baseline to other variables by treatment group. Up-arrows (↑) indicate a significant increase from baseline. Down-arrows (↓) indicate a significant decrease from baseline values. A ↔ indicates no difference compared to baseline values.

Variable	Treatment group (IU)	Change at week 1 vs. baseline	Change at week 4 vs. baseline	Change at week 12 vs. baseline
PTH	500 000	↔	↓	↔
	150 000	↓	↔	↓
	50 000	↔	↔	↓
VDBP & albumin	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
iCa & sCa	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
sCr & sPhos	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
FGF-23	500 000	↑	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↑	↔
24-hour urCa:urCr	500 000	↑	↑	↔
	150 000	↑	↑	↑
	50 000	↔	↔	↔
PINP	500 000	↑	↑	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
OC	500 000	↑	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
CTX	500 000	↑	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
SPPB	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Grip Strength	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Laying:standing pulse	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Laying:standing systolic blood pressure	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
Laying:standing diastolic blood pressure	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔
ARR	500 000	↔	↔	↔
	150 000	↔	↔	↔
	50 000	↔	↔	↔

Study strengths

This is the first study to try and elucidate the mechanism of why people might fall and fracture more after a single large bolus dose of vitamin D₃. A comprehensive range of biochemical and functional measurements have also been carried out to give a holistic assessment. Total 25(OH)D has been measured using more than one method, including measurement by the gold standard LC-MS/MS method. This is only the second study to report total 25(OH)D₃ measured by LC-MS/MS after a bolus dose of vitamin D₃ at ranges up to 500 000IU (only one previous study reported total 25(OH)D₃ measured by LC-MS/MS, but this was after a bolus of only 100 000IU).

This is also the first study to report serial measurements of free 25(OH)D and free 1, 25(OH)₂D after bolus dose vitamin D₃. A variety of methods have been used to measure and calculate free 25(OH)D in this study to give a comprehensive assessment of this metabolite. The consistency of the response profiles of the different vitamin D metabolites achieved across the different measurement methods for each metabolite is reassuring and gives confidence to conclusions made from the data.

This is also the first study to report the effects of a single large D₃ bolus on the ARR and the first study to report the effects of a vitamin D bolus on postural changes in blood pressure. This is the first study to show that a single oral bolus dose of up to 500 000IU does not appear to cause a disproportionate rise in free vitamin D metabolites compared to total vitamin D metabolites.

Study limitations

The study population was an older group who were vitamin D deficient at baseline, but otherwise healthy. This contrasts with other studies that have reported an increase in falls and fracture after large doses of vitamin D. In those studies participants have tended to be older and frailer than the population studied here and have a better baseline vitamin D status. We must consider the possibility that any adverse effects of large doses of vitamin D may only present in weaker population sub-groups.

Based on data from other bolus dosing studies that had previously been carried out and to reduce participant burden an initial post-administration time point of 5-7 days was determined as this was estimated to be the point in which we would see peak concentrations of total 25(OH)D. However, large increases have been reported as early as 3 days after a 600 000IU dose (173) and therefore it is plausible that we may not have captured the absolute peak total 25(OH)D and the peak concentrations of other metabolites.

The levels of total 25(OH)D achieved in this study after the largest bolus would appear to not be high enough to saturate VDBP in circulation, as animal studies and human studies of vitamin D intoxication have reported total 25(OH) levels of ~500nmol/l and above are required to achieve a displacement of free metabolites from the VDBP and to cause hypercalcemia (47, 225, 228, 316, 317). Despite this, the increases in total 25(OH)D that we have reported are certainly in line with those reported in other studies that had demonstrated adverse effects of large

doses of vitamin D. In the 500 000IU group the total 25(OH)D achieved is similar to thresholds that have been associated with adverse events in other studies (10, 223).

We did not measure DBP genotype and so it is possible that there are variations in the VDBP genotype in our participants that may give rise to different binding affinities affecting calculated free metabolite results. However, we used a Caucasian population only and measured VDBP with the superior polyclonal antibody assay, which limits variation in calculated free 25(OH)D. We also found that the calculated methods using the VDBP concentrations measured by the polyclonal assay were comparable to the measured free 25(OH)D. This is in line with reports from other studies (165).

Participants performed highly on the SPPB tests and this poses the possibility that the SPPB may not have been sensitive enough to detect any adverse effects on physical function in our relatively healthy cohort.

The study did not manage to recruit to target and did not have adequate power to detect changes in some outcome measures, but no trend was found in most secondary outcomes in response to the bolus doses.

Future directions

Participants performed highly on the SPPB tests and this poses the possibility that the SPPB may not have been sensitive enough to detect any adverse effects on physical function in our relatively healthy cohort. Future studies should investigate potential subtle effects of bolus dose vitamin D on motor function, gait, co-ordination and balance using wearable movement sensors (e.g. APDM/OPAL sensors) that may not have been picked up by the SPPB.

We cannot rule out a direct effect of the increase in vitamin D metabolites on the nervous system and brain. The cerebellum is essential for balanced muscular activity. Therefore, this region of the brain may be of interest when considering the effects of the mechanisms behind why bolus dose vitamin D₃ might cause falls in future research.

The ARR is affected by a wide variety of factors and as a result is a noisy variable (323). A larger sample size may be required to give the study adequate power to detect any treatment effect on the ARR (and some other secondary outcomes). Some protocols suggest that to improve sensitivity and reduce measurement variability a liberal salt diet should be encouraged and the collection of blood midmorning from seated patients following 2-4 hours upright posture improves sensitivity (323). The practicalities of the study did not allow for these procedures to be incorporated into the protocol, but future work should take this into consideration.

It is not clear if the transient increase in CTX immediately after bolus dosing that have been found in this study is clinically relevant, but this mechanism may help to explain the increase in fractures immediately after administration that has previously been reported and this also requires further investigation.

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