A systems biology approach to studying the effect of increasing vitamin D intake through food fortification on 25OHD status, in different population groups

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December 2015
Declaration of Originality

I confirm that the submitted work is my own work and that I have clearly identified and fully acknowledged all material that is entitled and to be attributed to others (whether published or unpublished) using the referencing system set out in the programme handbook. I agree that the University may submit my work to means of checking this, such as plagiarism detection service Turnitin® UK. I confirm that I understand that assessed work that has been shown to have been plagiarised will be penalised.

L.R. Wilson
Abstract

Vitamin D deficiency is a major public health concern in the UK. As the natural sources of vitamin D in the UK are limited, supplementation or food fortification are possible strategies for achieving the dietary recommendations of 10 μg/d that will be introduced in 2016 for the whole population.

However, there is controversy as to whether vitamin D$_2$ and vitamin D$_3$ are equally effective at raising vitamin D status (25OHD concentration). The primary and secondary aims of this PhD project were: to investigate the effects of both these forms of vitamin D independently on vitamin D status, markers of bone and cardiovascular health, and gene expression; as well as to examine whether common genetic variants affect response to either form of vitamin D.

A cohort of 90 South Asian and 245 Caucasian women were recruited onto a randomised-controlled trial; the D2-D3 Study. Participants were given either 15 μg/d of vitamin D$_2$, 15 μg/d of vitamin D$_3$ or placebo, in fortified foods, for 12 weeks.

At baseline, serum total 25OHD concentrations were significantly lower in the South Asian women (27.6 nmol/L) than the Caucasian women (60.3 nmol/L). In both the South Asian and Caucasian women, 25OHD concentrations significantly decreased in the placebo intervention (-5% and -15% respectively, p<0.001), and significantly increased in both the vitamin D$_2$ (112% and 39% respectively, p<0.001) and the vitamin D$_3$ interventions (243% and 72% respectively, p<0.001), with significantly greater increases seen in the vitamin D$_3$ intervention (p<0.001). In the vitamin D$_2$ groups, parathyroid hormone (PTH) concentrations decreased in the South Asian women (p<0.001), who had higher baseline concentrations, and were maintained in the Caucasian women, who had healthy baseline PTH concentrations. This effect was not seen with vitamin D$_2$ fortification. Over the 12 weeks, there were no clinically relevant changes in blood lipid concentrations in response to either vitamin D$_2$ or D$_3$, in the South Asian and Caucasian women.

Interestingly, whole blood transcriptome analysis indicated that the vitamin D$_2$ and D$_3$ interventions triggered a difference in expression of entirely different genes, and predicted therefore a difference in the activity of the respective metabolic and cellular pathways. The associations between genetic polymorphisms and change in 25OHD concentration in response to vitamin D also appear to differ depending on the form of vitamin D taken, although baseline 25OHD concentration may be a confounder.

The implications of this work, as the largest RCT conducted to date and showing conclusively that vitamin D$_3$ is more effective than vitamin D$_2$ at raising total 25OHD concentration and achieving or maintaining a healthy PTH concentration, are important: in the clinical setting vitamin D$_3$ may be preferable in the treatment of vitamin D deficiency. The novel findings that vitamin D$_2$ and vitamin D$_3$ lead to different metabolic/cellular responses requires further research to determine whether the response to vitamin D$_2$ is due to a decrease in 25OHD$_3$ concentration (observed in this study following vitamin D$_2$ treatment) or whether it is in response to the increase in 25OHD$_3$ concentration.
Statement of Contribution

Personal Contributions

• Participant recruitment and day-to-day running of the D2-D3 Study, alongside Dr Laura Tripkovic.
• Laboratory work: measurement of plasma lipid and vitamin D binding protein concentrations, and completion of the lab work within the gene expression Chapter alongside Dr Giselda Bucca.
• All statistical analysis presented, except the gene expression data handling and analysis.

General Contributions

• General supervision and proofreading of thesis manuscript: Professor Susan Lanham-New, Dr Kathryn Hart and Dr Laura Tripkovic.
• Serum vitamin D measurements: Dr Jacqueline Berry (University of Manchester, UK).
• Collection of blood samples from participants: Sue Starkey (phlebotomist), Haidee Shead (nurse) and Mary Gallagher (nurse).
• Recruitment of participants was supported by a) the Primary Care Research Network and b) Mrs Shanaz Buno (Surrey County Council).

Specific Chapter Contributions

Chapter 3 - The D2-D3 Study and Chapter 4 - The D2-D3 Bone Study

• Surrey Pathology Service (Frimley, Camberley, UK) measured the biochemical markers; parathyroid hormone, calcium, albumin, liver function tests, and urea and electrolytes.

Chapter 5 - The D2-D3 CVD Study

• Dr Francesca Robertson supported the use of the ILAB 650 (Instrumentation Laboratory, Milan, Italy) at the University of Surrey for the measurement of blood lipid concentrations.

Chapter 6 - The D2-D3 Genetic Study: Gene Expression

• General supervision of the work in, and proofreading of, this chapter: Professor Colin Smith, Dr Giselda Bucca and Dr Carla Möller-Levet.
• The laboratory work was completed alongside Dr Giselda Bucca at the University of Surrey.
• Data handling and statistical analysis of array data was completed by Dr Carla Möller-Levet.

Chapter 7 - The D2-D3 Genetic Study: Genetic Variants

• Selection of SNPs for inclusion and data analysis was supported by Professor Elina Hyppönen (University College London) and Ms Alana Cavadino (Queen Mary’s University London).
• LGC Genomics (Herts, UK) completed the DNA extraction and SNP genotyping laboratory work.
• Proofreading of chapter drafts: Dr Kourosh Ahmadi.

1 University of Surrey staff unless otherwise stated
Acknowledgements

Firstly thank you to Professor Susan Lanham-New, Dr Kath Hart and Dr Laura Tripkovic for their supervision and support throughout my PhD. I could not have asked for a better team to have been a part of over the past four years, and I look forward to many more years if we are lucky. I am so grateful to BBSRC DRINC for funding my studentship so that I could be a part of the D2-D3 Study team. Also thank you to everyone in the extended team - Dr Helen Lambert, Dr Andrea Darling, Taryn Smith, Saskia Wilson-Barnes - for making the office such an enjoyable place to be, and to everyone who helped with the D2-D3 Study days – Dr Caroline Bodinham, Ellie Nightingale, Sue Starkey, Haidee Shead and Mary Gallagher - for making all of those early mornings fly by.

My thanks also go to Professor Colin Smith, Professor Elina Hyppönen, Dr Giselda Bucca, Dr Carla Möller-Levet, Dr Ruan Elliott, Dr Kourosh Ahmadi and Miss Alana Cavadino for their help with the subject areas that were new to me. Your knowledge and expertise was invaluable.

Thank you to all of the participants on the D2-D3 Study, and to everyone who helped with recruitment; particularly Mrs Shanaz Bano who helped us recruit so many of our South Asian women.

To all of my beloved friends; thank you for putting up with my lack of appearance in recent times. You have all been so supportive and understanding, and you will never truly know how much I have appreciated your continued friendship.

My incredible family; thank you is just not enough. Mum and Dad, you have always been so supportive of Alia, Becky and I to follow whatever we want in life and we wouldn’t be who we are today if it were not for you. Alia and Becky, thank you for being the best sisters and friends I could ask for. Grandma and Grandad, thank you for showing me the importance of working hard, living life and loving, and how to strike the perfect balance. See you somewhere over the Rainbow, Grandad x

The biggest thank you of all is to my Marc. You have been so supportive and patient throughout my PhD, and kept me level headed in stressful times. Thank you for all the cuddles, for all the meals, for all the cups of tea and for looking after me. I could not have done this without you. 831 always x
Conference Proceedings, Abstracts and Future Publications

Selected Conference Proceedings and Abstracts
(The complete list can be found in Appendix 1)

1. Poster presentation at the 8th International Symposium on Nutritional Aspects of Osteoporosis, 17-19 May ‘12 (Lausanne, Switzerland)
   Wilson et al. (2012) Influence of Habitual Dietary Intake and Age on Risk of Poor Bone Health in Pre-Menopausal Women (Appendix 2)

2. Poster presentation at the 15th Vitamin D Workshop 19-22 June ‘12 (Texas, USA)
   Wilson et al. (2012) Vitamin D2 vs Vitamin D3 Food Fortification: Preliminary Baseline Results From A Randomised Controlled Trial in Caucasian/South Asian Women - The D2-D3 Study (Appendix 3)

3. Poster presentation at the Nutrition Society Summer Meeting 16-19 July ‘12 (Belfast, Ireland)

4. Oral presentation at the Nutrition Society Winter Meeting 11-12 Dec ‘12 (London, UK)

5. Oral presentation at the Nutrition Society Summer Meeting, 15-18 July ‘13 (Newcastle, UK)

6. Poster presentation at the BORS/BCOS Conference, Sept ‘13 (Oxford, UK)
   Wilson et al. (2013) Volumetric Bone Mineral Density and Dietary Patterns Across Three Age Groups of Caucasian and South Asian Women (Appendix 7)

7. Poster presentation at the Vitamin D and Human Health: from the Gamete to the Grave, April ‘14 (Queen Marys University London, UK)
   Wilson et al. (2014) The Effect of Vitamin D3 Supplementation on 25OHD Status, Blood Pressure and Blood Lipid Concentrations: A Pre-Menopausal vs. Post-Menopausal Comparison (Appendix 8)

8. Poster presentation at the Vitamin D Workshop, June ‘14 (Chicago, USA)
   Wilson et al. (2014) Does the presence of the metabolic syndrome impair the response to vitamin D fortification? A sub-analysis of the D2-D3 Study (Appendix 9)

9. Poster presentation at the Nutrition Society Summer Meeting, July ‘14 (Glasgow, UK)
10. Poster presentation at the National Osteoporosis Society Conference, Nov ‘14 (Birmingham)
Wilson, L.R., Tripkovic, L., Hart, K., Elliott, R., Smith, C.P., Bucca, G., Penson, S., Chope, G.,
Hypponen, E., Berry, J. and Lanham-New, S., 2014, November. Is vitamin D3 more effective
than vitamin D2 in raising 25OHD status in women with osteoporosis and osteopenia?
In Osteoporosis International (vol. 25, pp. S687-s687), England: Springer London Ltd.
(Appendix 11)

11. Poster presentation at the Vitamin D Workshop, April ‘15 (Delft, Netherlands)
Wilson et al. (2015) Ethnic variation in the associations between genetic variants and 25-
hydroxyvitamin D: The D2-D3 Study (Appendix 12)

12. Poster presentation at the International Symposium on the Nutritional Aspects of
Osteoporosis, June ‘15 (Montreal, Canada)
(Appendix 13)

Invited Review

Lanham-New SA and Wilson LR. 2016. Vitamin D - has the new dawn for dietary recommendations
14)

Future Publications and Papers in Preparation

R, Hyppönen E, Berry JL and Lanham-New SA. Vitamin D3 - Conclusively Superior to Vitamin D2 in
Raising Status: A Randomised Trial. Under Review

Wilson et al. Effect of vitamin D2 and D3 supplementation, via fortified foods, on bone health in
South Asian and Caucasian women (PTH concentrations, and CTX subject to future work)

Wilson et al. Effect of vitamin D2 and D3 supplementation, via fortified foods, on blood lipids in
South Asian and Caucasian women

Wilson et al. Differences in gene expression following 12 weeks of 15 μg/d vitamin D2 vs. vitamin D3
supplementation

Awards

Prize Winner – Oral presentation at the Biosciences KTN ECR Food Sector, 23rd May ’13 (London, UK)
Vitamin D Intakes and Blood Pressure in Caucasian and South Asian women

Awarded a University of Surrey Bursary to attend The Times Cheltenham Science Festival 2014

Awarded a BBSRC Policy Internship at the Parliamentary Office of Science and Technology (POST)
following a written application and interview day. During this internship at POST I completed a
briefing report for MPs and Parliamentarians on Diet in Pregnancy and Breastfeeding
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Important note: In this thesis the term ‘South Asian’ will be used to denote individuals whose ethnic origin is that of the South Asian sub-continent (Bangladeshi, India, Pakistan) as well as the Arabian Peninsula (United Arab Emirates, Saudi Arabia, Oman, Yemen). The term ‘Caucasian’ will be used throughout as shorthand for ‘white Caucasian’.
Chapter 1 - INTRODUCTION
1.1 General Introduction

Over the past decade, vitamin D has become a focus of attention and great interest not only within the scientific, clinical and academic communities, but also government and regulatory organisations, industry, the media and the general public. This stems from the growing knowledge base and concerns about the prevalence of low vitamin D status worldwide, as well as the concomitant discovery that this vitamin has many functions beyond its classical, well established role in regulating calcium and phosphorus homeostasis for musculoskeletal health. These functions include regulation of immune function, hormone secretion, cell proliferation and cell differentiation (Bikle 2009), as a result of the active form of vitamin D binding to the vitamin D receptor (VDR) in a large number of cells to promote or suppress gene transcription and thus regulate cell function. Some of these identified functions of vitamin D may begin to explain how, in addition to vitamin D status being associated with bone health, low vitamin D status has also been associated with several non-skeletal disorders including cancer, heart disease, diabetes, high blood pressure, age-related cognitive decline, Parkinson’s disease, multiple sclerosis and arthritis. However, causal associations with these disorders have not been established (Autier et al. 2014).

There is a general consensus that a vitamin D status (serum 25OHD) <25 nmol/L should be classified as deficient. However, there are differences in opinions as to what the optimal vitamin D status should be. As a result of this, there are also differences in opinions as to what the optimum dose of vitamin D is in order to achieve optimal vitamin D status.

Vitamin D is unique, in that the main source in the UK is not the diet, but rather the sub-cutaneous synthesis of vitamin D under the surface of the skin on exposure to ultraviolet B (UVB) rays from sunlight. In the UK, the UVB rays required for the synthesis of vitamin D are only present between the months of April-September. Dietary contributions to vitamin D status are also limited by the lack of available foods which are a good source of vitamin D. Previously, it was though that sufficient vitamin D was made during the summer months from sun exposure to maintain sufficient 25OHD concentrations during the winter, however it is now clear that this is not necessarily the case and there are many individuals who are vitamin D deficient all year round, and not just in the winter. According to the latest National Diet and Nutrition Survey (NDNS), 24% of men and 22% of women in the UK are known to have a low vitamin D status (25OHD <25 nmol/L) year round, increasing to 39% of men and women in the winter (Bates et al. 2014). This is not a unique problem to the UK; across Europe, vitamin D deficiency has been reported in 2-30% of European adults (Spiro & Buttriss 2014). In the UK there is also particular concern about ‘at-risk’ groups such as South Asian women in whom vitamin D deficiency is extremely common, as high as 81% in the winter (Darling et al. 2013a).
It is not just the availability of sources of vitamin D that affect vitamin D status, as there are several other factors that do so too, including age, skin pigmentation, adiposity and genetics.

1.1.1 Current Relevance

In the summer of 2015 the UK Government’s Scientific Advisory Committee on Nutrition (SACN) published a draft report for consultation on *Vitamin D and Health* (SACN, 2015) proposing the introduction of dietary reference values for all age groups, not just those considered as vulnerable as previously. A reference nutrient intake (RNI) of 10 μg/d has been proposed for the UK population over 4 years of age as the amount needed for 97.5% of the population to maintain serum 25OHD above 25 nmol/L when UVB exposure is minimal. This brings the UK into alignment with many other countries in the world who have had dietary recommendations for some time. The next step will be to determine how the UK population can achieve this dietary recommendation, as obtaining 10 μg/d from natural food sources alone would be difficult and vitamin D deficiency is already a concern across the UK. Possible strategies could include fortification of foods or supplementation. There are two main forms of vitamin D - vitamin D$_2$ and vitamin D$_3$ – that can be used in supplementation or fortification. Vitamin D$_3$ is the form made from UVB exposure, whereas both vitamin D$_2$ and vitamin D$_3$ are obtained from a few foods in the diet. There is ongoing controversy as to whether both vitamin D$_2$ and vitamin D$_3$ are equally effective at raising vitamin status (Tripkovic *et al.* 2012). Clear data showing whether the two forms of vitamin D are equally effective at raising 25OHD levels and improving health outcomes associated with serum 25OHD such as bone health and cardiovascular health, given the equivocal research findings to date, will provide key information for policy makers and industry members in the development of policies or strategies for improving vitamin D status and health. Determining mechanisms by which vitamin D$_2$ and vitamin D$_3$ may be differentially effective, by measuring vitamin metabolites and gene expression in response to the two forms, as well as assessing whether individuals genetic variants affect response to supplementation, would all be key to building a greater understanding of the potential differences or similarities between vitamin D$_2$ and vitamin D$_3$.

1.2 Vitamin D Biology and Metabolism

1.2.1 Structure

Vitamin D is classified as a secosteroid, as the molecular structure is similar to steroid hormones such as cortisol and aldosterone, due to the cyclopentane perhydro phenanthrene ring structure and the presence of a broken carbon-carbon bond within one of these rings (Norman 2008).
overarching term used to describe both vitamin D$_2$ and vitamin D$_3$, also known as ergocalciferol and cholecalciferol respectively. These two forms of vitamin D have two differences in their chemical structure, as shown in Figure 1-1, with vitamin D$_2$ (C$_{28}$H$_{44}$O) having an additional double bond between carbon atoms 22 and 23 and an additional methyl group on carbon atom 24, than vitamin D$_3$ (C$_{27}$H$_{44}$O). These differences give vitamin D$_2$ a 3.1% higher molecular mass than vitamin D$_3$ (396.65 g/mol and 384.64 g/mol respectively). Unless otherwise specified, the term ‘vitamin D’ refers to both vitamin D$_2$ and vitamin D$_3$ throughout this thesis.

![Figure 1-1 The structure of vitamin D$_2$ and vitamin D$_3$. Adapted from Holick, 2007](image)

### 1.2.2 Sources

Although called a vitamin, vitamin D is not technically a vital amine. It is rather a pro-hormone that can be produced within the body and it is therefore not an essential dietary factor. Both vitamin D$_2$ and D$_3$ can be obtained from foods or dietary supplements. Vitamin D$_2$ is formed by exposure of ergosterol (a plant steroid) to UVB and is therefore formed in plants, whereas vitamin D$_3$ is formed cutaneously by the action of UVB on 7-dehydrocholesterol and is therefore found in animal products and is the only form the human body can make itself.

#### 1.2.2.1 Cutaneous Synthesis

Vitamin D$_3$ is synthesised from 7-dehydrocholesterol (7-DHC) that is found in the epidermis and dermis of the skin of humans and animals. The action of sunlight, containing UVB radiation of wavelengths 290-315nm, on the skin converts 7-DHC to pre-vitamin D$_3$ (MacLaughlin et al. 1982) by the breaking of the 9,10 carbon-carbon bond. Pre-vitamin D$_3$ is then converted to a more
thermodynamically stable vitamin D$_3$ by a temperature dependent isomerization reaction (Bickle 2009). This cutaneously produced vitamin D$_3$ enters the extracellular fluid before diffusing into dermal capillaries (Holick 2011) via the plasma vitamin D binding protein which preferentially translocates vitamin D$_3$ from the skin into the circulation (Holick 1981).

1.2.2.2 Dietary Sources

There are a few naturally rich food sources of vitamin D, mostly of animal origin containing vitamin D$_3$ (egg yolk, oily fish), although vitamin D$_2$ is found naturally in wild mushrooms (Matitilda et al. 1994; Teichmann et al. 2007). Both vitamin D$_2$ and D$_3$ are also commercially synthesised for use as supplements and to fortify food, by UVB irradiation 7-DHC from sheeps’ wool and ergosterol from plants and fungi, respectively (Bickle 2009).

1.2.3 Metabolism

1.2.3.1 Absorption of Dietary Vitamin D

Both vitamin D$_2$ and D$_3$, ingested through the diet are lipid soluble and are taken-up by passive diffusion in the small intestine, into enterocytes (Haddad et al. 1993). In the enterocyte they are incorporated into chylomicrons and transported by the lymphatic system into the venous circulation. In continued association with chylomicrons, it is then transported to the liver by the vitamin D binding proteins (VDBP) or lipoproteins (DeLuca 2004).

1.2.3.2 Conversion of Vitamin D to the Active Metabolite

Both vitamin D$_2$ and D$_3$, irrespective of source, go through the same two-step hydroxylation process to become the biologically active form of vitamin D: 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), also known as calcitriol.

Once in the circulatory system both vitamin D$_2$ and vitamin D$_3$ bind to the VDBP and are transported to the liver (Holick 2005). In the liver, both vitamin D$_2$ and D$_3$ are converted to 25-hydroxyvitamin D (25OHD) via the action of 25-hydroxylases (25-OHase), such as those from the cytochrome P450 (CYP) group (CYP2R1 and CYP27A1). 25OHD is the major circulating metabolite measured to determine vitamin D status (section 1.3.1). Following hydroxylation in the liver, 25OHD then binds to VDBP in the circulation and is transported to the kidney. In the kidneys, 25OHD is converted to 1,25(OH)$_2$D, by 25-hydroxyvitamin D-1-α-hydroxylases (1α-OHase), such as CYP27B1 (DeLuca 2004). This critical step is under the tight homeostatic regulation of circulating parathyroid hormone (PTH) concentration (Deeb et al. 2007). The available 1,25(OH)$_2$D is then able to elicit the biologic functions of vitamin D.
1.2.3.3 Degradation and Excretion of Vitamin D

For both vitamin D₂ and vitamin D₃ metabolites, hydroxylation of both 25OHD and 1,25(OH)₂D, by 24-hydroxylase enzymes (24-OHase) such as CYP24A1, is the first step of degradation, as shown in Figure 1-2 for vitamin D₃ (DeLuca 2004). Following a series of four successive reactions catalyzed by CYP24A1 an inactive water-soluble compound is produced, which is then excreted in bile (Henry 2011).

![Figure 1-2](image_url)

Figure 1-2 The metabolism pathway of vitamin D₃ and the proposed roles of 1,25(OH)₂D. Taken from Deeb et al. (2007).

1.2.3.4 Differences in Metabolism of Vitamin D₂ and D₃

Both vitamin D₂ and vitamin D₃ maintain their difference in the side chain throughout metabolism, and so vitamin D₂ is converted to 25OHD₂ and then 1,25(OH)₂D₂, and vitamin D₃ is converted to
25OHD$_3$ and then 1,25(OH)$_2$D$_3$ (Jones et al. 1998). The pathway for vitamin D$_3$ is shown in Figure 1-2. Although vitamin D$_2$ undergoes the same metabolic processes to vitamin D$_3$, it is unclear if all details of regulation and biological activity are identical to those of vitamin D$_3$ (Henry 2011). There have been reports that the differences in the side chains between the two forms of vitamin D directly affects the rate of vitamin D$_3$ conversion to serum 25OHD, as it is thought that vitamin D$_2$ is the preferred substrate for hepatic 25-hydroxylase (Holmerg et al. 1986; Cheng et al. 2003; Cheng et al. 2004). Vitamin D$_2$ and its metabolites also have a lower binding affinity to DBP than vitamin D$_3$ and its metabolites (Houghton & Vieth 2006).

In the degradation and excretion of vitamin D, once 1,24,25(OH)$_3$D has been formed, the vitamin D$_2$ metabolite, 1,24,25(OH)$_3$D$_2$ is deactivated and is irretrievable (Horst et al. 1986). However, when vitamin D$_3$ goes through the same process and 1,24,25(OH)$_3$D$_3$ is formed it retains its capacity to bind to the VDR (Houghton & Vieth 2006) and requires a further process for deactivation (Horst et al. 1986). This extra step gives vitamin D$_3$ the potential to remain biologically active for a greater period, therefore maintaining vitamin D status for a longer period than vitamin D$_2$.

1.3 Physiological Role of Vitamin D

1.3.1 Mechanisms of Physiological Role

Circulating 1,25(OH)$_2$D diffuses across cell membranes and enters the nucleus where it binds to the vitamin D receptor (VDR). Bound to the VDR, which is a ligand-activated transcription factor, 1,25(OH)$_2$D elicits its biological function through the regulation of gene transcription and by activating signal transduction pathways (Norman et al. 1992).

The VDR is expressed in enterocytes, osteoblasts, parathyroid and distal renal tubule cells; all cells involved in calcium and phosphate homeostasis (Jones 1998), which supports the classic, physiological role of vitamin D in calcium and phosphate homeostasis and bone mineralization. The VDR is also found in a wide range of other cells and tissues not previously thought of as target tissues for vitamin D, including leucocytes, macrophages, pancreatic β-islet cells, ovarian tissue, prostate, placenta and adipose tissue (Jones 1998; Norman 2008). The discovery of the VDR in a wide variety of cells and tissues has led to the recognition that the VDR, and therefore vitamin D, has a role to play in many more processes than previously thought. Many cells that express VDR also have the enzyme CYP27B1 and therefore they have the capacity to produce 1,25(OH)$_2$D, without 25OHD having to be transported to the kidney (Bickle 2009).
Both 1,25(OH)\(_2\)D\(_2\) and 1,25(OH)\(_2\)D\(_3\) appear to be similar in their binding affinity to the VDR (Hollis 1984), however VDR has a higher affinity for 1,25(OH)\(_2\)D\(_3\) (Norman 2008).

1.3.2 Skeletal Role - Calcium and Phosphorus Regulation

The major biological function of the active form of vitamin D, 1,25(OH)\(_2\)D (also known as calcitriol), is to maintain serum calcium and phosphorus concentrations within the narrow normal ranges of 2.2-2.6 mmol/L and 0.8 to 1.4 mmol/L respectively. Calcium and phosphorus homeostasis is essential for bone mineralization (DeLuca 2004), and calcium homeostasis is also important for neuromuscular function (Holick 2011). As shown in Figure 1-3, serum calcium is tightly regulated (Rasmussen & DeLuca, 1963) and if calcium-sensing transmembrane proteins in the parathyroid gland detect a decrease in calcium, parathyroid hormone (PTH) is secreted immediately. This release of PTH induces the production of 1,25(OH)\(_2\)D in osteoblasts and proximal convoluted tubule cells in the kidney, which then stimulates intestinal calcium absorption, renal calcium absorption and bone resorption to increase serum calcium concentration. The opposite response is also seen, with the parathyroid gland reducing PTH secretion when an increase in calcium concentration is detected (Holick, 2007).

The efficiency of calcium absorption from the intestine decreases from 30-50% to no more than 15% when the body becomes vitamin D insufficient (Holick 1996). Vitamin D can also promote bone matrix formation by increasing resorption through osteoclast activity, however, this direct effect of vitamin D on bone is only present in calcium or vitamin D deficient conditions (Underwood and DeLuca, 1984; Elsman and Bouillon, 2014).
Figure 1-3 Schematic representation of the role of 1,25(OH)2D on calcium, phosphorus, and bone metabolism. Vitamin D, either ingested in the diet or produced in the skin by exposure to UVB radiation, is converted in the liver to 25OHD, by 25-hydroxylase (25-OHase). 25OHD is then converted in the kidneys to 1,25(OH)2D, by 1-OHase. 1,25(OH)2D increase calcium (Ca2+) and phosphorus (HPO42-) absorption in the intestine, and stimulates the release of Ca2+ and HPO42- from bone as result of stimulating the expression of RANKL on the osteoblasts which interacts with RANK on preosteoclasts, inducing mature osteoclastic activity. Adapted from Holick 2006.

1.3.3 Non-skeletal Roles

The discovery that the vitamin D receptor is expressed in virtually all cells in the body (Norman 2008), and not just cells and tissues that would be considered targets of vitamin D action, has led to the recognition that the VDR and therefore vitamin D, has a role to play in many more processes than previously thought. This concurs with the growing body of observational data supporting associations between serum 25OHD and diseases such as cancer, cardiovascular disease and diabetes. Evidence has so far shown that the non-calcioem actions of vitamin D, through the actions of the VDR-ligand, include cell proliferation, cell differentiation and immune-modulatory functions (Holick 2006; DeLuca 2004; Ingraham et al. 2008; Verstuyf et al. 2010).
1.4 Vitamin D Classification

1.4.1 Measurement of Serum 25OHD Concentration

Serum 25OHD is the most useful measurement for assessing individuals vitamin D status (DeLuca 2004; Seaman & Cashman 2009), as the concentration of the biologically active form of vitamin D (1,25(OH)₂D) is homeostatically regulated (DeLuca 2004; Holick 2006). Serum 25OHD also has a half-life of about 2-3 weeks (Lund et al. 1980), whereas 1,25(OH)₂D has a half-life of 7 hours (Lips 2007).

There are several different methods available for measuring 25OHD, including competitive protein binding assay (Belsey et al. 1974), high-performance liquid chromatography (Eisman et al. 1977), radioimmunoassay (Hollis and Napoli, 1985), enzyme immunoassay (Lind et al. 1997) and the more recent assays based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Maunsell et al. 2005). Different methodologies and laboratories have shown variation in the concentration of serum 25OHD measured (Binkley et al. 2004; Lips et al. 1999), and the lack of standardised measurements of 25OHD is a possible explanation for why there are inconsistencies and controversies, nationally and internationally, as to what concentration of 25OHD defines vitamin D ‘deficiency’, ‘insufficiency’ and ‘optimum’ and which method should be used (Spiro and Buttriss 2014).

In recent years, LC-MS/MS has become the gold standard reference method for 25OHD concentration and is now being actively used by a number of key organisations, including the US Centre for Disease Control and Prevention (CDC) and the UK National Laboratories. In 2010, the US National Institutes for Health (NIH) established the Vitamin D Standardization Program (VDSP) in an attempt to standardise the laboratory measurement of vitamin D around the world. In addition to this, the Vitamin D External Quality Assessment Scheme (DEQAS), which has been led by the UK since 1989, now has greater prominence. DEQAS call for 75% or more of the results to fall within ± 25% of the target value (the National Institute of Standards and Technology LC-MS/MS assigned value) in order to achieve a proficiency certificate.

Despite advances in methodology for the assessment of vitamin D status, and the consistency of laboratory measurements improving with standardised and strict guidelines in place in many research centres, data collected prior to and outside of such guidelines may be unreliable and should be interpreted with caution (Binkley et al. 2014). Immunoassays have the advantage of being automated and therefore have a higher throughput than the LC-MS/MS. With increasing clinical
demand for 25OHD measurements, the automated immunoassays are a time saving option. However, LC-MS/MS enables more reliable analysis of 25OHD in comparison, and the ability to quantify the major circulating metabolites; 25OHD$_2$ and 25OHD$_3$ (Shah et al. 2011).

1.5 Optimal Vitamin D Status and Requirements

1.5.1 Optimal 25OHD Concentration

Internationally, there is a lack of consensus and definition on the suggested thresholds (cut-offs) used to define 25OHD concentration, from deficiency to optimal status, and the range of terminology and associated values used, make comparisons of reported prevalence difficult (Thatcher & Clarke 2011). The World Health Organisation and SACN agree that 25OHD concentrations <25 nmol/L (or 10 mg/ml) are the deficiency threshold (WHO 2003; SACN 2007; SACN 2015), with regard to the prevention of rickets and osteomalacia, and the WHO also define vitamin D insufficiency as 25OHD <50 nmol/L. However, the US Institute of Medicine (IOM) say that 25OHD <30 nmol/L is deficiency, 30-50 nmol/L is inadequacy, >50 nmol/L is sufficient (IOM 2011), and the UK National Osteoporosis Society (NOS) recently agreed with the IOM thresholds and proposed that the UK practitioners should also adopt them (Francis et al. 2013). Most typically, vitamin D deficiency is marked by a threshold of <25-30 nmol/L and insufficiency by 25OHD concentrations in the range of 25-49 nmol/L (Spiro and Buttriss 2014), however, the Endocrine Society Task Force (USA/Canada) define deficiency as 25OHD <50nmol/L and advocate that 25OHD concentrations should exceed 75 nmol/L (Holick et al. 2011).

Within the UK, the conflicting thresholds between SACN and the NOS are likely to cause differences in the treatment individuals receive, dependent on the guidelines their NHS Clinical Commissioning Group base their treatment protocols on. For example, if an individual presented themselves with a 25OHD concentration of 28 nmol/L in Derby they would be classed as ‘deficient’ as they use of a cut-off of <30 nmol/L (Southern Derbyshire, 2014). However, if they presented themselves in Surrey then they would not be ‘deficient’ as they use a cut-off of <25 nmol/L (NHS Surrey and Sussex, 2013).

In 2005, Dawson-Hughes and colleagues highlighted the differences in opinions of researchers in the field of vitamin D, specifically in relation to the optimal 25OHD concentration for fracture prevention (Dawson-Hughes et al. 2005). Using this outcome measure they reported ‘optimal’ concentrations ranging from 50-80nmol/L, as shown in Table 1.1.
In a review of evidence from studies that evaluated thresholds for 25OHD concentration in relation to bone mineral density (BMD), lower-extremity function, dental health, and risk of falls, fractures, and colorectal cancer they found that the most advantageous 25OHD concentration for all health outcomes was at least 75 nmol/L, but was optimal at 90-100 nmol/L (Bischoff-Ferrari et al. 2006).

Despite one-off studies and individual researchers suggesting optimal 25OHD concentrations on the higher range of 70-100 nmol/L, many organisations and researchers consider a desirable concentration of 25OHD to be above 25 nmol/L, due to the limited number of randomised controlled trials investigating higher concentrations, and a lack of long-term safety data (Lanham-New et al. 2011).

In this thesis the following terminology and thresholds will be used, unless otherwise stated; 
<25 nmol/L: deficient; 25-50 nmol/L: insufficient; 50-75 nmol/L: sufficient; >75 nmol/L: optimal

1.5.2 Dietary Recommendations
As there is no consensus among researchers on the optimal concentration of 25OHD for health, there is also variation in opinions across the world as to the dietary vitamin D intakes required to reach an optimal 25OHD status with suggested daily doses ranging from 10-40 μg/d (Dawson-Hughes et al. 2005).

1.5.2.1 UK Recommendations
Prior to the recent SACN Draft Report in 2015 (SACN 2015), there had been no dietary vitamin D recommendations in the UK for the general population aged 4-65 years (Table 1.2), as the recommendations had not been changed since 1991 when it was assumed that free-living individuals aged 4-65 years attained adequate body stores of vitamin D from cutaneous synthesis during the summer, to sustain 25OHD concentrations during the winter months (DoH 1991). However, this is

Table 1.1 Estimates of the minimum serum 25OHD concentration optimal for fracture prevention

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Optimal 25OHD Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lips</td>
<td>50 nmol/L</td>
</tr>
<tr>
<td>Holick</td>
<td>75 nmol/L</td>
</tr>
<tr>
<td>Heaney</td>
<td>80 nmol/L</td>
</tr>
<tr>
<td>Meunier</td>
<td>75 nmol/L</td>
</tr>
<tr>
<td>Vieth</td>
<td>70 nmol/L</td>
</tr>
<tr>
<td>Dawson-Hughes</td>
<td>80 nmol/L</td>
</tr>
</tbody>
</table>

Source: Dawson-Hughes et al. 2005
now known not to be the case for many individuals in the UK (Hyppönen & Power, 2007; Darling et al. 2013a). In 2007 SACN reviewed the recommendations, but made no changes as they were awaiting further definitive evidence from randomised-controlled trials (RCTs) in order to consider changes to existing dietary reference values (SACN 2007).

Cashman and colleagues published one of those awaited RCTs in 2008. They aimed to establish the distribution of dietary vitamin D required to maintain the serum 25OHD concentration of >97.5% of the population above the 25nmol/L deficiency threshold (Cashman et al. 2008). The study was conducted at two centers located at 51-55° N in Ireland. 238 healthy adults (aged 20-40 yrs) received 0, 5, 10 or 15µg/d of vitamin D₃ for 22 weeks over the winter period, with serum 25OHD measured at both baseline and the end of the study. The authors concluded that an average requirement of 8.7 µg/d of vitamin D was sufficient for >97.5% of the population, whilst 2.7 µg/y was sufficient for 90% of the population, to maintain 25OHD >25 nmol/L. In order to achieve 25OHD >50 nmol/L in 50%, 90% and 97.5% of the population, an estimated dietary requirement of 10.2 µg/d, 21.7 µg/d and 28 µg/d was sufficient, respectively.

The newly proposed UK recommendations (Table 1.2) are now more in line with other European and International recommendations, as the UK was previously the only country in Europe not to have any recommendations for 4-65 year olds, unless they were considered ‘at-risk’ of vitamin D deficiency.

### Table 1.2 Reference Nutrient Intakes (RNI) for vitamin D (µg/day) at different life stages

<table>
<thead>
<tr>
<th></th>
<th>Children</th>
<th>Adults</th>
<th>Older Adults</th>
<th>Pregnancy</th>
<th>Lactation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>United Kingdom</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹UK 1991</td>
<td>8.5</td>
<td>7</td>
<td>-</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>²UK 2015*</td>
<td>8.5-10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>European</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>³Austria/Germany/Switzerland*</td>
<td>10</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>⁴Belgium</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10-15</td>
<td>10-15</td>
</tr>
<tr>
<td>⁵France</td>
<td>20-25</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>⁶Ireland</td>
<td>7.0-8.5</td>
<td>10</td>
<td>0-10</td>
<td>0-15</td>
<td>0-10</td>
</tr>
<tr>
<td>⁷Spain</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
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<tr>
<td>⁸The Netherlands</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
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<tr>
<td>⁹NNR</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>¹⁰EC</td>
<td>7.0-8.5</td>
<td>10</td>
<td>0-10</td>
<td>0-15</td>
<td>0-10</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>¹¹IOM</td>
<td>10</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>¹²WHO/FAO</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>¹³NHMRC</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

1.6 Vitamin D Status in the UK

Low vitamin D status is a common problem across the UK, with particular sub-groups of the population, such as South Asian women, showing higher levels of deficiency (Darling et al. 2013a).

The latest National Diet and Nutrition Survey (NDNS) for the period 2008/9 – 2011/2 reported that 24% of men and 22% of women in the UK were vitamin D deficient (25OHD <25 nmol/L) year round, which increased to 39% of men and women between January and March (Bates et al. 2014). Previous NDNS data published in 2003 found that around 15% of the adult population were vitamin D deficient (Henderson et al. 2003) and so comparing these data sets would suggest that the prevalence of vitamin D deficiency in the UK has increased.

Seasonal variation in 25OHD concentration exists, as also shown in the latest NDNS data, with winter and spring showing lower concentrations as a result of the lack of UVB availability for the endogenous production of vitamin D$_3$. A cross-sectional study conducted by Hyppönen & Power (2007) measured 25OHD concentrations in 7,437 white British men and women at 45 years of age from the 1958 British birth cohort, and showed that during the winter and spring months 25OHD concentrations were deficient (<25 nmol/L), insufficient (<40 nmol/L) and sufficient (<75 nmol/L) in 15.5%, 46.6% and 87.1% of participants respectively. Figure 1-4 shows both seasonal and geographical variation in the prevalence of 25OHD concentrations below 40 nmol/l in Great Britain (Hyppönen & Power 2007).

Large seasonal fluctuation in 25OHD concentrations have been shown to be detrimental to bone health, showing associations with increased concentrations of serum parathyroid hormone, and C-terminal telopeptide (CTX - a bone resorption marker) (Darling et al. 2014), as well as urinary N-telopeptide of collagen (uNTX – a bone resorption marker) (Darling et al. 2015).
1.6.1 Current Status in UK South Asian Populations

In the UK the Asian/Asian British population make up 7.5% of the total population, the largest contribution of all ethnic minority groups (Office for National Statistics 2012). South Asian women in particular possess a cluster of the risk factors associated with an increased risk of vitamin D deficiency, including decreased skin synthesis due to darker skin pigmentation (Chapter 1.8.2.1) and reduced skin exposure to sunlight, either through lifestyle factors or clothes coverage for cultural reasons.

A study in Birmingham compared plasma 25OHD concentration in South Asians with non-Asians (white and Afro-Caribbean). This study had a total of 240 adults aged 17-98 years, with equal numbers of South Asians to non-Asians, and equal numbers of male to females. They found that in both summer and winter, South Asians had lower plasma 25OHD concentration than non-Asians. Only 18% and 6% of South Asians had concentrations >30 nmol/l in summer and winter respectively (Pal et al. 2003). A recent longitudinal study (D-FINES) looking at dietary and sunlight contributions to seasonal vitamin D status in post-menopausal women included a sub-set of South Asian women based in Surrey (n35). Using <25 nmol/l as a measure of deficiency, the authors found that 51.4% of Surrey South Asian women were deficient compared to 0% of Surrey Caucasian women in the summer. Those proportions rose to 64.5% and 9.5% respectively, in the winter. The median 25OHD for the South Asian women was 24 nmol/L in the summer and 16.9nmol/L in the winter (MacDonald et al. 2011; Darling et al. 2013a). In this D-FINES Study, both South Asian and Caucasian women were
recruited, and as shown in Figure 1-5, the South Asian women had consistently lower 25OHD concentrations year round, and were below the deficiency cut-off of 25 nmol/L almost year round. Interestingly, the fluctuations in 25OHD concentrations shown in the Caucasian populations may have a more negative impact on bone health than the consistently low 25OHD concentrations seen in the South Asian cohort (Darling et al. 2014; Darling et al. 2015).

**Figure 1-5** Serum 25OHD concentrations of both South Asian and Caucasian women in the D-FINES Study, at each season

Key: Pre: pre-menopausal; Post: post-menopausal. Provided by and included with permission from Andrea Darling (Darling et al. 2013a).

### 1.7 Dietary Sources of Vitamin D

Although sunlight is known to be the main source of vitamin D for the majority of the population, it is not an available source during the winter and spring months across the UK (Chapter 1.8.2.3). During these seasons dietary intakes of vitamin D become particularly important.

#### 1.7.1 Food Sources

There are only few foods available in the diet that can be considered a good natural source of vitamin D, with oily fish considered to be the best source. Table 1.3 shows some of the major sources of vitamin D in the UK diet. However, as shown in Table 1.3, even the same food source can vary greatly in vitamin D content with farmed salmon found to have 75% less vitamin D than wild salmon (Lu et al. 2007). Although little is known about the effect of cooking on vitamin D content, frying salmon in vegetable oil instead of baking it has been shown to lead to a 50% lower vitamin D content (Lu et al. 2007).
Table 1.3 Vitamin D content of various foods

<table>
<thead>
<tr>
<th>Food Source</th>
<th>Vitamin D (µg/100g)</th>
<th>D2 or D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon, wild</td>
<td>15-25</td>
<td>D3</td>
</tr>
<tr>
<td>Salmon, farmed</td>
<td>2.5-5</td>
<td>D3</td>
</tr>
<tr>
<td>Mackerel, flesh only</td>
<td>8.0</td>
<td>D3</td>
</tr>
<tr>
<td>Tuna, canned</td>
<td>6.0</td>
<td>D3</td>
</tr>
<tr>
<td>Sardines, flesh only</td>
<td>4.0</td>
<td>D3</td>
</tr>
<tr>
<td>Eggs, chicken</td>
<td>3.2</td>
<td>D2 / D3</td>
</tr>
<tr>
<td>Pork leg joint</td>
<td>0.9</td>
<td>D3</td>
</tr>
<tr>
<td>Lamb chop</td>
<td>0.8</td>
<td>D3</td>
</tr>
<tr>
<td>Shiitake mushrooms – sun-dried</td>
<td>40</td>
<td>D2</td>
</tr>
<tr>
<td>Shiitake mushrooms - fresh</td>
<td>2.5</td>
<td>D2</td>
</tr>
</tbody>
</table>

Source: McCance and Widdowson 2002; SACN 2007; DH 2013

1.7.2 Supplements
Vitamin D supplements are available in both vitamin D$_2$ and vitamin D$_3$ forms. Fish oils, such as cod liver oil are also a supplemental source of vitamin D$_3$. Circulating 25OHD concentrations have been shown to be significantly higher in participants who use vitamin D supplements, including fish oil supplements, than in those who do not (Hyppönen & Power 2007).

Although supplement use is more common practice in Scandinavian countries such as Norway, in the UK it is less common (Calvo et al. 2005). Only 13% of men and 20% of women from the 1958 British Birth Cohort Study at age 45 years use cod liver oil, fish oil or other supplements containing vitamin D (Hyppönen & Power 2007), which supports figures previously reported in the National Diet and Nutrition Survey (12% and 24% respectively) (Henderson et al. 2003). However, in the updated NDNS survey this appears to have increased to 25% in men, but remained at 24% in women (Bates et al. 2014). The most recent Infant Feeding Survey has also highlighted that vitamin D supplement uptake is poor among expectant mothers, with only 3% of mothers recalling taking a vitamin D supplement despite schemes such as the Health Start Scheme (McAndrew et al. 2012).

1.7.3 Vitamin D Food Fortification
Supplements could be an effective method for individuals to increase their intake of vitamin D, but clearly only if they are taken. There are several factors that may lead to individuals not taking supplements such as cost issues, access and availability to supplements and/or lack of awareness of
the importance. This is where food fortification represents an opportunity to increase vitamin D supply on a population wide basis.

In 1940 it became mandatory to fortify margarine and fats spreads with a fat content above 80% with vitamin D in the UK in response to the evidence that a large proportion of the population were at risk of deficiency (SACN 2007). The amount of vitamin D added is relatively low, as the purpose of fortification was to increase the vitamin D content to that naturally occurring in butter. However, in 2014 this mandatory requirement was revoked (DEFRA 2014), although manufacturers have continued to voluntarily fortify foods. In the US, Canada (Calvo et al. 2004) and more recently Finland, (Tylavsky et al. 2006) fortification of milk with vitamin D has become mandatory.

Both vitamins D\textsubscript{2} and D\textsubscript{3} can be used to fortify foods, via UVB irradiation of ergosterol obtained from yeast or 7-dehydrocholesterol obtained from sheeps’ wool-derived lanolin respectively (Holick 2007). Several manufacturers in the UK are beginning to fortify their products with vitamin D on a voluntary basis, particularly breakfast cereals and yoghurts. However, cases of hypervitaminosis D caused by over-fortification of milk in the US (Jacobus et al. 1992; Blank et al. 1995) highlights the need for clear fortification plans and monitoring of those plans.

1.8 Factors Affecting Vitamin D Status

1.8.1 Factors Affecting Dietary Intakes of Vitamin D

With limited dietary sources of vitamin D, achieving an adequate intake through diet alone would take considerable attention to the types and quantities of foods consumed. The NDNS Survey data shows mean dietary intakes of vitamin D in British men and women to be 3.9 and 3.4μg/d respectively (Bates et al. 2014), with approximately 0.8 μg of this, for both the men and women, coming from fortified foods such as meats and breakfast cereals.

High consumption of oily fish is the most likely route to achieving requirements through natural dietary sources alone (Table 1.3), although intakes would need to be very high to achieve 10 μg/d ie. 2 tins of sardines (250g), or a tin of tuna (160g), or a 200-400g portion of salmon every day. However, the current advice for oily fish is one portion (140g) per week and only 23% of adults aged 19 to 64 are even consumers of oily fish (Bates et al. 2014).
In addition to absolute intakes via the diet, issues of bioavailability may also need to be considered. Vitamin D obtained from the diet, referring to both D$_2$ and D$_3$, is lipid soluble and it has been speculated that vitamin D may require other lipids to not only stimulate the release of bile acids in the duodenum to facilitate lipid absorption (Basu et al. 2003), but also with which to associate in chylomicrons to facilitate its absorption transportation into the circulation. The efficiency of vitamin D absorption is approximately 50% in the presence of malabsorptive disease (Shinchuck & Holick 2007), but this has been shown to be even lower when dietary fat intake is low (Ovesen et al. 2003).

A recent interest in the effects of dietary fat composition on vitamin D absorption has emerged and although there is very little evidence for this, a two-year supplementation trial published in 2011 found that the change in plasma 25OHD in response to supplemental vitamin D$_3$ was positively associated with monounsaturated fatty acid (MUFA) intakes and negatively associated with polyunsaturated fatty acid (PUFA) intakes (Niramitimahapanya et al. 2011). However, the authors based fat intake on a food frequency questionnaire for which they were not able to define the fat content or composition of any specific meal and so further confirmatory research is required before fat-specific recommendations can be made.

### 1.8.2 Factors Affecting the Cutaneous Synthesis of Vitamin D$_3$

#### 1.8.2.1 Skin Pigmentation

Darker skins tones contain more melanin, which effects the cutaneous production of vitamin D$_3$, as it is a natural filter of UVB rays. Some research has shown that at a set UVB exposure the production of 25OHD is slower in darker skin (Clemens et al. 1982), with paler skin said to accrue vitamin D up to ten times faster than darker skin (Holick 2004).

#### 1.8.2.2 Age

The epidermal levels of 7-dehydrocholesterol remain relatively constant until later in life when levels begin to decline (Holick & Garabedian 2006), with a 70 year old only able to produce a quarter of the amount of vitamin D$_3$ as a 20 year old from the same sun exposure (Holick 2004).

#### 1.8.2.3 Season and Latitude

As shown in Chapter 1.6, seasonal variation in plasma 25OHD concentrations are observed in the UK, with higher levels of deficiency in winter and spring months (Hyppönen & Power 2007; Darling et al. 2013a). Webb et al. (1988) first revealed the effect that season and latitude have upon the production of previtamin D$_3$. In the UK, at latitudes of 50-60°N, from mid-October through to the
beginning of April the UV radiation does not allow for the cutaneous synthesis of vitamin D₃ as it is of an inappropriate wavelength (Webb et al. 1988). Even within the UK, the difference in latitude affects 25OHD concentrations (Hyppönen & Power 2007; Macdonald et al. 2011). For the remaining months when UVB rays are available, 60% of the effective UV radiation occurs between 11:00-15:00 hours. Only when the length of a shadow is shorter than the object casting it is the sun at an angle at which cutaneous synthesis of vitamin D can occur (Holloway 1990). Individuals therefore rely solely on dietary intakes and stores in adipose tissue during the winter and spring seasons, which have now been shown to be insufficient for the majority of the UK population for maintaining serum 25OHD concentrations (Hyppönen & Power 2007; MacDonald et al. 2011; Darling et al. 2013a; Bates et al. 2014).

1.8.2.4 Other Environmental Factors

Atmospheric pollution (Agarwal et al. 2002), cloud cover and the ozone layer can all have a limiting factor on the proportion of UVB rays available to skin (Engleson 2010).

1.8.2.5 Avoidance of the Sun, Use of Sunscreen and Skin Covering

One of the factors recognised by Hyppönen & Boucher (2010) as being contributory to the low vitamin D concentrations seen in the UK is the recognised link between sunlight exposure and skin cancer risk. For cancer protective purposes and following advice previously released by the Cancer Research UK SunSmart campaign, avoidance of sun exposure and the regular use of sunscreen is common, significantly reducing the synthesis of vitamin D (Matsuoka et al. 1987; Webb & Engelsen 2008). In 2010 a Consensus Vitamin D Position Statement was produced to represent the views of the British Association of Dermatologists, Cancer Research UK, Diabetes UK, the Multiple Sclerosis Society, the National Heart Forum, the National Osteoporosis Society and the Primary Care Dermatology Society (2010), where cancer protective sun avoidance was considered in combination with the need for some sun exposure for vitamin D requirements. However, whilst it stresses sunburn avoidance, as opposed to total sun avoidance, this distinction has not necessarily been clearly communicated to the wider public (Lanham-New et al. 2011).

Dress style is also an important factor influencing absorption of UVB rays, as clothing reduces surface area of skin exposed to sunlight and has been shown to prevent vitamin D production (Matsuoka et al. 1992), and may go some way in providing a possible reason as to why population groups such as South Asian women have high levels of deficiency (Macdonald et al. 2011; Darling et al. 2013a).
1.8.3 Adiposity

Vitamin D can be stored in body fat, and so it is thought that greater adiposity may lead to greater storage of vitamin D and subsequently a poorer vitamin D status. This relationship between adiposity and vitamin D status has been shown in several studies. Direct comparisons between obese and non-obese subjects has shown lower serum 25OHD concentrations in the obese subjects (Hyppönen & Power 2007), and lower 25OHD with increasing obesity has also been shown (Beydoun et al. 2010). In healthy subjects total body fat as measured by whole body dual energy x-ray absorptiometry scan (Snijder et al. 2005) and bioelectrical impedance analysis (Looker 2005) has also been shown to be inversely associated with plasma 25OHD concentrations. The influence of body fat on vitamin D status may also explain some of the differences seen in ethnic groups, as body compositions vary between ethnic groups.

1.8.4 Genetics

With heritability, as a determinant of circulating 25OHD, estimated to range between 29-80% in previous family and twin studies (Shea et al. 2009; Arguelles et al. 2009; Wjst et al. 2007; Hunter et al. 2001), it has been suggested that genetic factors play a greater part than personal, social and cultural factors that determine sun exposure and diet. Shea and colleagues (2009) showed that non-genetic determinants, such as reported vitamin D intake and season of measurement, only account for about a quarter of the inter-individual variability in 25OHD concentration. More recent work has therefore aimed to identify genetic variants affecting vitamin D concentrations, and there have been several key genome-wide association studies and systematic reviews that have begun to identify single nucleotide polymorphisms, associated with vitamin D metabolism, that are linked to vitamin D status. These studies, and the associations between genetics and vitamin D status, are discussed in Chapter 7.

1.9 The D2-D3 Debate

There are mixed opinions amongst vitamin D experts, and controversy in the evidence, as to whether vitamin D₂ and vitamin D₃ are both equally effective in raising 25OHD status. Based on a number of trials published in the 1930s, it had been assumed for some time that vitamin D₂ and D₃ were equally effective at raising 25OHD concentrations in humans (Park 1940). However, over the past two decades controversy has arisen. There are at least three studies looking at the comparative efficacy of vitamin D₂ and vitamin D₃ at raising 25OHD concentrations in an intervention trial that have shown findings that support the earlier evidence that vitamin D₂ and D₃ are equally effective (Holick et al. 2008; Biancuzzo et al. 2010; Fisk et al. 2012), but the findings from at least ten other intervention
trials that have directly compared the two forms, have suggested that vitamin D₃ is superior to D₂ in raising 25OHD (Tjellsesen et al. 1986; Trang et al. 1998; Armas et al. 2004; Romagnoli et al. 2008; Glendenning et al. 2009; Binkley et al. 2011; Heaney et al. 2011; Logan et al. 2013; Lehmann et al. 2013; Oliveri et al. 2015). Given that food fortification and supplements may contribute greatly to vitamin D status when sunlight exposure is not a viable source of vitamin D, it is important that this issue is resolved.

In 2012, a systematic review and meta-analysis of the comparative efficacy of vitamin D₂ vs D₃ on 25OHD concentrations in randomised, controlled trials was conducted (Tripkovic et al. 2012). Although ten studies were identified as having a direct comparison of vitamin D₂ to D₃ within a randomised controlled trial, only seven studies had sufficient data for inclusion in the meta-analysis (Trang et al. 1998, Romagnoli et al. 2008, Holick et al. 2008, Glendenning et al. 2009, Biancuzzo et al. 2010, Heaney et al. 2011, Binkley et al. 2011). When all of the studies were combined, regardless of dosing frequency, vitamin D₃ showed a significantly greater effect in the raising of serum 25OHD concentrations than the D₂, as shown in Figure 1-6 (Tripkovic et al. 2012). When only analysing those studies that gave vitamin D₂ vs. D₃ as a bolus dose (Romagnoli et al. 2008, Heaney et al. 2011, Binkley et al. 2011) the meta-analysis continued to show a significantly greater effect in the raising of 25OHD concentration over time for vitamin D₃ as a bolus dose than for D₂. However, when the studies that gave vitamin D₂ vs. D₃ on a daily basis were combined (Trang et al. 1998, Holick et al. 2008, Glendenning et al. 2009, Biancuzzo et al. 2010, Binkley et al. 2011) there was no significant difference between D₂ and D₃ interventions in the raising of serum 25OHD concentrations.
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Figure 1-6 Forest plot indicating absolute change in 25OHD from baseline comparing the effects of vitamin D3 with that of D2.

Random-effects meta-analysis comparing the effects of daily and bolus supplementation of D3 with that of D2 on net changes in serum 25OHD concentrations. Forest plot indicating the absolute change in serum 25OHD concentrations from baseline favored the D3 intervention. ∆25(OH)D denotes the change in serum 25OHD concentrations from baseline, squares denote mean differences [with 95% CIs (lines)], and Total denotes the cumulative n from all included studies. With the use of a random-effects model, overall, there was a significant greater effect in the raising of serum 25OHD concentrations over time for D3 supplementation compared with D2. (Tripkovic et al. 2012)

1.9.1 Potential Mechanisms for Difference in Efficacy of Vitamin D2 and D3

Evidence behind the differential effects of vitamin D2 and D3 in raising serum 25OHD concentrations has been accumulating. Although both vitamin D2 and vitamin D3 are similar in molecular structure, the additional double bond and methyl group essentially means they are different chemicals. As discussed in Chapter 1.2.3.4, it is thought that vitamin D3 is the preferred substrate for hepatic 25-hydroxylase and is therefore converted to 25OHD before vitamin D2 (Holmerg et al. 1986; Cheng et al. 2003; Cheng et al. 2004). It is also thought that the additional methyl group on carbon 24 of vitamin D3 increases its affinity for vitamin D binding protein (VDBP) and the vitamin D receptor (VDR) (Houghton & Vieth 2006). This also appears to be linked to an additional step in 24-hydroxylation that inactivates 1,25(OH)2D. Once 1,24,25(OH)3D2 has been formed, vitamin D2 has been deactivated and is irretrievable (Horst et al. 1986). However, when vitamin D3 goes through this process and 1,24,25(OH)3D3 is formed, it retains its capacity to bind to the VDR (Houghton & Vieth 2006) and requires a further process for deactivation (Horst et al. 1986). This gives vitamin D3 the potential to remain biologically active for a greater period than vitamin D2.

Some of these suggestions have been partially explored in clinical trials, with evidence showing a quicker production of 25OHD, and higher concentrations sustained for longer, in response to vitamin D3 than D2 (Heaney et al. 2011; Armas et al. 2004). Furthermore, Heaney et al. (2011) noted far
greater degradation of serum 25OHD$_2$ than serum 25OHD$_3$ over 6 weeks after 12 weeks of weekly bolus dosing.

Despite this agreement between mechanistic studies showing that vitamin D$_3$ appears to have advantageous qualities over vitamin D$_2$ for hydroxylation to 25OHD, and the clinical trial evidence that has shown that vitamin D$_3$ increases 25OHD to higher concentrations and for longer than vitamin D$_2$, there remains contrasting evidence showing an equal efficacy of vitamin D$_2$ and D$_3$ (Holick et al. 2008; Biancuzzo et al. 2010; Fisk et al. 2012), as was also shown in the meta-analysis when all daily dosing studies were pooled (Tripkoivc et al. 2012). However, previous studies have been flawed (Lanham-New et al. 2010), with under powering within treatment arms, and heterogeneity within the meta-analysis was high.

1.10 Summary of Thesis Rationale

There is no doubt that vitamin D deficiency and insufficiency are real concerns across the UK at present, particularly for individuals from ethnic minority populations with darker skin pigmentation and much less sun exposure, such as South Asian women. They are likely to be at risk of deficiency year round, whereas Caucasian women in the UK are more at risk of deficiency during the winter and spring months, and carry the additional risk associated with vitamin D fluctuations. As natural dietary sources of vitamin D are lacking in the UK, and individuals cannot rely on the sun exposure in the winter and spring months, supplementation and food fortification are viable options for achieving the newly proposed RNI of 10 μg/d (SACN 2015). Policy makers, food manufacturers and health professionals need a conclusive answer as to whether vitamin D$_2$ and vitamin D$_3$ are equally effective at improving vitamin D status in order to ensure effective strategies for improving vitamin D status are in place.

As well as determining whether vitamin D$_2$ and vitamin D$_3$ are equally effective at raising 25OHD concentrations, and the possible mechanisms that may explain differences in their efficacy, it would also be beneficial to determine whether both forms have the same effect on bone parameters, as the classical role of vitamin D is in bone health, and also on cardiovascular disease parameters, which have previously been associated with 25OHD concentrations but lack randomised-controlled trial data showing causality. These are introduced in greater depth and justified in Chapter 4 and Chapter 5, respectively.
With the discovery of non-classical roles of vitamin D, such as immune function and cell proliferation, it would also be interesting to identify the effects of vitamin D$_2$ and vitamin D$_3$ on gene expression to identify whether they affect the same pathways and elicit the same response in gene expression. This research is introduced and justified in Chapter 6.

If fortification were to be considered as an approach to tackling vitamin D deficiency as a population wide approach, knowing that the intervention would work effectively across the population would be key. The importance of assessing the impact of genetic variants on 25OHD concentrations or response to supplementation, via fortification, is introduced in Chapter 7.
Chapter 2 - METHODOLOGY
2.1 The D2-D3 Study

2.1.1 Study Design

The D2-D3 Study was run within the Clinical Investigation Unit (CIU) at the University of Surrey over two consecutive winter periods; October 2011- March 2012 and October 2012-March 2013. The study was a randomised, double blind, placebo-controlled parallel food fortification trial. For a 12-week trial period participants were asked to consume specially prepared intervention products (an orange juice and a biscuit) daily, alongside their normal diet, through which they would receive 15µg/d of vitamin D$_2$, 15µg/d of vitamin D$_3$ or placebo. The five intervention groups were: placebo, vitamin D$_2$ juice, vitamin D$_2$ biscuit, vitamin D$_3$ juice and vitamin D$_3$ biscuit. Participants were given both and orange juice and a biscuit to consume daily, of which only one would be fortified and the other would be placebo, except in the case of the placebo group where both products were placebo. Participants visited the CIU three times over the course of the 12-weeks; baseline, week 6 and week 12. A small sub-set of participants were also invited to a fourth visit at week 16, four weeks after the intervention had been completed.

2.1.2 Recruitment

Participants were recruited onto the study through collaboration with the Primary Care Research Network (PCRN). The PCRN are a national research network for primary care, part of the National Institute for Health Clinical Research Network. They provide support for researchers and health professionals involved in primary care research studies. They contacted GP practices within the Surrey Primary Care Trust with information about the D2-D3 Study to request the GP surgeries support in recruitment. In the surgeries that collaborated, the research team screened the patient database for suitable potential participants and letters of invitation (Appendix 15) were sent to those patients, along with a study information sheet (Appendix 16). In addition to the recruitment via the PCRN, the research team also made use of local media advertisement (posters, newspapers and radio), and visited local businesses and community groups. Participants were also recruited from databases and networks established during the University of Surrey based D-FINES study (NHS REC No.O6/Q1090/1) and from a research database available at the Surrey Clinical Research Centre (University of Surrey, Guildford, UK).

2.1.3 Screening

Participants were screened prior to taking part in the study, using the Health & Lifestyle Questionnaire (Appendix 17) to ensure that they met the inclusion criteria shown in Table 2.1.
Table 2.1 Inclusion and exclusion criteria for the D2-D3 Study

<table>
<thead>
<tr>
<th>Inclusion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Female</td>
<td></td>
</tr>
<tr>
<td>- White Caucasian or South Asian (Indian/Bangladeshi/Pakistani/Arabian Peninsula) ethnicity</td>
<td></td>
</tr>
<tr>
<td>- Aged 18-65 years</td>
<td></td>
</tr>
<tr>
<td>- Premenopausal or at least three years post-menopausal</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Exclusion</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>- Prior or present history of: ischaemic heart disease; type 1 or type 2 diabetes; thyroid disease; osteoporosis, osteopenia or other musculoskeletal disease; rheumatoid arthritis; haematological disease; malignancy; gastro-intestinal disorders; liver or kidney disease; clinical depression or other psychological disorders; eating disorders; drug or alcohol abuse within the last 2 years</td>
<td></td>
</tr>
<tr>
<td>- Taking prescribed steroids or other medications likely to affect vitamin D metabolism, or to interact with vitamin D supplementation</td>
<td></td>
</tr>
<tr>
<td>- Currently receiving treatment for medical conditions likely to affect vitamin D metabolism</td>
<td></td>
</tr>
<tr>
<td>- Abnormal liver or kidney function</td>
<td></td>
</tr>
<tr>
<td>- Smoking &gt;20 cigarettes a day</td>
<td></td>
</tr>
<tr>
<td>- Alcohol intake &gt;14 units a week</td>
<td></td>
</tr>
<tr>
<td>- Currently involved in a clinical trial</td>
<td></td>
</tr>
<tr>
<td>- Sunbed use or a sun holiday within three months prior to commencing the trial, or during the study period</td>
<td></td>
</tr>
<tr>
<td>- Taking vitamin D supplements and not able/willing to refrain from use for three months prior to and during the study period</td>
<td></td>
</tr>
<tr>
<td>- Following a weight reducing diet or under dietary restriction (except vegetarianism)</td>
<td></td>
</tr>
<tr>
<td>- Have known intolerance/allergies to the constituent ingredients of the intervention products</td>
<td></td>
</tr>
</tbody>
</table>

2.1.4 Ethical Consideration

In accordance with the ethical standards laid down in the 1964 Declaration of Helsinki, a favourable ethical opinion was obtained from the South-East Coast (Surrey) NHS Research Ethics Committee (NHS REC No. 11/LO/0708) (Appendix 18-22) and the University of Surrey Ethics Committee (Fast-Track EC/2011/97/FHMS) (Appendix 23-26). The study protocol can be found in Appendix 27. Written, informed consent was obtained from all subjects (Appendix 28) prior to participation.

2.1.5 Sample size and Power Calculation

The study required a minimum of 320 subjects (at 90% power) for recruitment into the five key intervention groups. This has been calculated as follows: 240 Caucasian women (n 48 in each of the five groups) and 80 South Asian women (n 16 in each group). These will be randomly recruited to one of five intervention groups, shown in Figure 2-1.

Using data from published research papers (Trang et al. 1998; Holick et al. 2008; Cashman et al. 2008; Cashman et al. 2009) and response of serum total 25OHD concentrations to vitamin D$_2$ or D$_3$ as the primary end-point, it was calculated that these study numbers were required to: (i) detect a 0.6 SD size effect at 90% power in Caucasian women; (ii) detect a 1.1 SD size effect at 90% power in
South Asian women. To allow for a 10% drop-out rate, a recruitment target of 355 women (265 Caucasian women and 90 South Asian) was set.

### 2.1.6 Randomisation

Participants were allocated to one of five possible intervention groups (Figure 2-1) via a randomised allocation system using a computer-generated randomisation programme. The randomisation was stratified to take into account the participants’ ethnicity, BMI and age, and was verified by the trial statistician with the codes assigned to the participants by a trial investigator (the investigator was blinded to the randomisation). The trial statistician was responsible for keeping the code.

**Figure 2-1 D2-D3 Study Trial Flowchart**

The D2-D3 Study trial flowchart shows target recruitment numbers and the five intervention groups that participants are randomly assigned to.
2.1.7 Intervention Products

An orange juice (liquid food product) and a biscuit (solid food product) were selected as the intervention food products and vehicles for vitamin D fortification, following an assessment of commonly consumed foods in both South Asian and Caucasian women.

The intervention products were developed and produced by Campden BRI (Chipping Campden, UK). The juice and biscuit were designed to have a comparable nutritional value, particularly in terms of calories and calcium (Table 2.2). Camden BRI then produced three batches of juices and three batches of biscuits; placebo, vitamin D₂ fortified and vitamin D₃ fortified. The vitamin D₂ and vitamin D₃ for the fortification products were both manufactured by Lycored (Kent, UK). Microencapsulated vitamin D₂ or D₃ (750 μg/kg [30000 IU/kg]) was added to biscuit dough to provide 15 μg [600IU] per 20g dough piece, and 17g baked biscuit. Cold-water soluble vitamin D₂ or D₃ (73 μg/kg [2927 IU/kg]) was added to each batch of orange juice to provide 15 μg [600IU] per 205g serving of orange juice.

The amount and stability of vitamin D₂ and D₃ in the orange juice and biscuits was determined using HPLC MS/MS. The products were found to contain either no vitamin D (placebo) or vitamin D₂ or D₃ within 10% of their specified concentrations. Concentration of vitamin D₂ and D₃ was found to be stable after storage at room temperature for three months.

The manufacturers blinded intervention products during the process of packaging.

### Table 2.2 Nutritional composition of the solid and liquid fortification products, per serving

<table>
<thead>
<tr>
<th>Amount per serving</th>
<th>Juice</th>
<th>Biscuit</th>
</tr>
</thead>
<tbody>
<tr>
<td>kCal (g)</td>
<td>73.1</td>
<td>76.8</td>
</tr>
<tr>
<td>Ca (mg)</td>
<td>17.2</td>
<td>15.7</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>0.9</td>
<td>1.0</td>
</tr>
<tr>
<td>Total Carbohydrates (g)</td>
<td>17.6</td>
<td>10.6</td>
</tr>
<tr>
<td>Of which sugars</td>
<td>15.3</td>
<td>3.5</td>
</tr>
<tr>
<td>Fat</td>
<td>0.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Saturated fat</td>
<td>0.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Dietary fibre (g)</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>Sodium (mg)</td>
<td>17.2</td>
<td>44.9</td>
</tr>
<tr>
<td>Water (g)</td>
<td>12.6</td>
<td>0.4</td>
</tr>
<tr>
<td>Potassium (mg)</td>
<td>260.7</td>
<td>15.3</td>
</tr>
<tr>
<td>Iron (mg)</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>
2.1.8 Study Protocol

Over the 12-week study period participants were required to attend three visits – a baseline visit at 0 weeks/baseline, a week 6 visit and a week 12 visit – as shown in Figure 2-2. Study visits took place in the morning (between 7am and 11am) at the Clinical Investigation Unit (CIU) based at the University of Surrey. For the purpose of the blood samples, participants were requested to attend fasted, following a 12-hour overnight fast (ie. fasting from 8pm for a 8am appointment).

Figure 2-2 Study timeline of visits and study activities at each visit

2.1.8.1 Screening Process

A screening questionnaire (Health & Lifestyle Questionnaire, Appendix 17) was completed with the participants to assess their suitability for the study. Study appointments were then arranged with the participants and an appointment letter was sent out 2 weeks prior to their appointment, along with a diet diary (Appendix 30) and instructions on how to complete this (Appendix 29).

2.1.8.2 Baseline/Week 0

At the baseline visit participants were given the opportunity to ask any questions before informed consent was obtained. Participants had their blood pressure measured and gave a fasted blood sample for measurement of serum 25OHD and parathyroid hormone. This blood sample was also used to perform a full blood count that day, to check haemoglobin level for undiagnosed anaemia that would exclude the participant from continuing on the study. After blood samples were obtained, participants were given breakfast. At this study visit anthropometric measurements and a peripheral Quantitative Computed Tomography (pQCT) scan of the non-dominant forearm (radius) were also performed. Their 4-day food diary was also collected in and checked for consistency with the participant. Participants were then given their 6-week provision of randomly assigned intervention
products and a dosimeter (to be returned via sealed addressed envelope provided), and follow-up details were arranged.

Between appointments participants were contacted fortnightly to help maintain good compliance, and to confirm the next appointment arrangements.

### 2.1.8.3 Week 6

At this second visit an adverse events and compliance interview was completed with the participant (Appendix 31), and anthropometric measurements, blood pressure and a fasted blood sample were taken as they were at the baseline visit. Participants were offered breakfast and provided with their next 6-week provision of assigned intervention products.

Between appointments participants continued to be contacted fortnightly to help maintain good compliance and to confirm week 12 appointment arrangements. An appointment letter was sent out 2 weeks prior to the appointment with another blank 4-day food diary for completion, and a new dosimeter to wear for 4 days, prior to appointment.

### 2.1.8.4 Week 12

At this third appointment an adverse events and compliance interview was completed again, and a dosimeter and 4-day diet diary were checked and collected in. Anthropometrics and blood pressure were measured, and a fasted blood sample taken as previously. Participants were then given breakfast.

A sub-set of participants were invited to attend a fourth visit, 4 weeks later. Participants were asked dependent on compliance and date of the third visit (so that fourth visit took place before the end of March).

### 2.1.8.5 Week 16 in Sub-Set

In a sub-set of participants invited to a fourth visit (week 16), anthropometrics, blood pressure and a fasted blood sample were taken. Participants were then offered breakfast.

### 2.1.9 Data Collection Procedures

#### 2.1.9.1 Anthropometric Measurements

At each appointment, anthropometric measurements were collected, as detailed below.
**2.1.9.1.1 Height**
Height was measured via a wall-mounted Harpenden Stadiometer (Holtain Ltd, Crymych, Dyfed). Participants stood with their shoes off, heels together, maintaining an up-right posture. Measurements were taken to the nearest 0.1cm.

**2.1.9.1.2 Weight, BMI and Body Fat Percentage**
Participants’ body mass and composition were measured using a Tanita TBF-300 Body Composition Analyser (Tanita Europe BV, Netherlands) in accordance with the manufacturer’s instructions. Prior to use the machine was set up for each individual participant; 1.0kg was deducted to take into account the weight of clothing, and information on their gender, age and whether they were ‘athletic’ or ‘normal’ build was also entered. An athlete is classified as a person involved in intense physical activity for approximately 12 hours per week or more, those who belong to a sport team or a sport organisation with the aim of participation in competition, those who exercise to build up muscles like a bodybuilder or those who are professional athletes. Once set, participants were requested to remove socks and shoes and stand on the scales for approximately 15 seconds until the scales had stabilised and recorded a measurement. Each measurement included data on body fat percentage, total body water, fat free mass and estimated basal metabolic rate, with reference ranges for a healthy population.

**2.1.9.1.3 Waist & Hip Circumference**
Waist and hip circumferences were measured using a flexible tape measure, to the nearest 0.1cm. Measurements of waist circumference were taken directly around the navel and hip circumference was measured around the widest part of the hips using the same tape measure. Waist:Hip ratio (WHR) was calculated as waist measurement (cm) divided by hip measurement (cm).

**2.1.9.1.4 Blood Pressure**
Participants rested in a supine position for 10 minutes in a quiet room. An OMRON 705IT Auto Arm Blood Pressure Monitor (Omron Healthcare, Kyoto, Japan) was then used to take the blood pressure from the non-dominant arm in accordance with the manufacturer’s instructions. This was repeated three times, with the mean systolic and diastolic blood pressure calculated from these readings.

**2.1.9.2 Bone Density**
A peripheral quantitative computed tomography (pQCT) scan was completed for each of the participants to measure bone density, as discussed below.
2.1.9.2.1 pQCT
A peripheral quantitative computed tomography (pQCT) scanner (XCT 3000, Stratec Medizitechnik GmbH, Germany) was used to perform a scan of the radius, to assess volumetric bone mineral density (vBMD) and bone strength of the non-dominant arm. Bone strength is not only determined by bone density, but also bone geometry (size and thickness of cortex). pQCT uses x-rays to image slices through the radius to calculate size, mass and density, including that of the cortical and trabecular bone layers separately, allowing the measurement of vBMD which is not confounded by bone size.

2.1.9.3 Dietary Intakes and UVB Exposure
To assess the contributions of diet and UVB exposure to vitamin D status, diet diaries were completed and dosimeter badges were worn by participants, as detailed below.

2.1.9.3.1 Diet Diaries
Participants were asked to complete two four-day food diaries over the study period; at 0 weeks and 12 weeks. They were provided with a food diary that contains photographs to aid with portion estimation and were asked to include at least one weekend day (Appendix 29-30). The diet diaries were analysed using DietPlan (Version 6, Forestfield Software Ltd, Horsham, UK), following a departmental Standard Operating Procedure (SOP) (Appendix 32).

2.1.9.3.2 Dosimeter Badge
Participants were requested to wear a dosimeter (a polysulphone film badge) on their outer clothing, in the collar to shoulder area, when outdoors for seven consecutive days at the beginning of the 12 weeks, and another badge again at the end of the 12 weeks. This allowed for measurement of ambient UVB exposure.

The dosimeter badges were read before and after wear, using a spectrophotometer at the University of Surrey (CECIL Aquarius CE72000). The following formula was used to convert absorbance at 330nm, as read by the spectrophotometer, into Standard Erythemal Dose (SED), an erythemally weighted measure of radiant exposure equivalent to 100 Jm⁻², to get a reading in SED for each individual at baseline and week 12 of the study:

\[
\text{SED} = 10.7 [\Delta A_{330}] + 14.3 [\Delta A_{330}]^2 - 26.4 [\Delta A_{330}]^3 + 89.1 [\Delta A_{330}]^4
\]
2.1.9.4 **Compliance**

At both follow-up appointments, at week 6 and 12, participant compliance to the study was formally monitored by interviews and packet counts (Appendix 31). Regular telephone contact (minimum fortnightly) assisted in encouraging and monitoring participant compliance through the duration of the study.

2.1.9.5 **Blood Sample Collection and Processing**

A vacutainer system (Beckton Dickinson Diagnostics, USA) was used for all venepuncture by a trained phlebotomist. The order of draw was as follows: 10ml SST tube, 6ml EDTA tube, 3ml EDTA tube then 6ml EDTA tube.

The following blood collection tubes were used (all supplied by Beckton Dickinson Diagnostics, USA).

- A 10ml BD Vacutainer® SST™ tube
  - Collects: Serum
  - Determinations for: total 25OHD, 25OHD₂, 25OHD₃, 1,25(OH)₂D, calcium
  - Preservative: For 5ml draw tube – Clot activator/polymer gel
- A 3ml and two 6ml BD Vacutainer® Ethylenediaminetetraacetic Acid (EDTA) tubes
  - Collects: Plasma
  - Determinations for: parathyroid hormone, albumin, lipids, vitamin D binding protein
  - Preservative: Dipotassium Ethylenediaminetetraacetic Acid (K₂EDTA)
    - For 3ml draw tube – 5.4mg K₂EDTA
    - For 6ml draw tube – 10.8mg K₂EDTA

All samples collected were inverted ten times and then transferred to the laboratory in the CIU for processing immediately. The 3ml EDTA sample was not processed, as the whole blood sample was used to measure full blood count using a Haemotology Analyser. The remaining samples were processed as per the manufacturer’s instructions. The EDTA tubes were centrifuged immediately at 1751 x g (3000rpm) for 10 minutes at 4°C (Labofuge 400R – Kendro Laboratory Products, Germany). The SST tubes were allowed to clot at room temperature for 1 hour and then centrifuged, also at 1751 x g (3000rpm) for 10 minutes at 4°C. Post-centrifugation, the samples were aliquoted using disposable pasteur pipettes (liquipette - Elkay Laboratory Products (UK) Ltd, Basingstoke, UK) into assigned 2ml Apex® Plus Screw-cap microtubes (Alphalabs, Hampshire, UK) at volumes of approximately 1-0.5ml per microtube. The samples were promptly frozen at -20°C for 24 hours and then at -80°C thereafter.
2.1.10 Biochemical Analysis

Surrey Pathology Service (Frimley, Camberley, UK) measured the biochemical markers; parathyroid hormone (PTH), calcium, albumin, liver function tests (LFTs), and urea and electrolytes (U’s & E’s). The procedures for PTH, calcium and albumin measurements are detailed below.

2.1.10.1 Parathyroid Hormone

Plasma intact PTH was measured using a two-site sandwich chemiluminescent immunoassay using the ADVIA Centaur XP Immunoassay System (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK). Manufacturer’s quoted inter- and intra-assay CVs are 3.4% and 4.0% respectively.

2.1.10.2 Calcium

Serum calcium was measured using an endpoint spectrophotometric reaction based on the o-cresolphthalein complexone (CPC) methodology using the ADVIA 2400 Chemistry System with a (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK). Manufacturer’s quoted inter- and intra-assay CVs are 1.9% and 1.1% respectively.

2.1.10.3 Albumin

Serum albumin was measured using an endpoint spectrophotometric reaction based on the bromocresol green solution (BCG) dye binding methodology using the ADVIA 2400 Chemistry System with a (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK). Manufacturer’s quoted inter- and intra-assay CVs are 1.3% and 0.6% respectively.

2.1.11 Vitamin D and Metabolites

Total serum 25-hydroxyvitamin D (25OHD) concentrations were measured by Dr Jacqueline Berry (University of Manchester and Manchester Royal Infirmary, UK). The method used could distinguish between 25-hydroxyvitamin D₂ (25OHD₂) and 25-hydroxyvitamin D₃ (25OHD₃) components of total 25OHD, allowing the quantification of these metabolites independently too. Serum 25OHD concentrations were determined by liquid chromatography tandem mass spectrophotometry (LC-MS/MS) using an ABSciex 5500 tandem mass spectrophotometer (AB Sciex UK Ltd, Warrington, UK) and the MassChrom® 25OHD3/D2 kit for LC-MS/MS (Chromsystems Instruments and Chemicals GmbH, Gräfelfing, Germany) following the manufacturers’ instructions (laboratory intra- and inter-assay CV 3.7% and 4.8% respectively). The laboratory is accredited by the Clinical Pathology
Accreditation (CPA) UK (CPA number 0865) and has been certified as proficient by the Vitamin D Quality Assurance Scheme (DEQAS).

### 2.1.11.1 1,25-dihydroxyvitamin D

1,25-dihydroxyvitamin D (1,25(OH)₂D) was measured using the DiaSorin LIAISON® XL 1,25 Dihydroxyvitamin D, which is an in vitro chemiluminescent immunoassay (CLIA). This was completed by The Doctors Laboratory (The Doctors Lab Ltd, London, UK) in serum samples. Manufacturer’s quoted inter- and intra-assay CVs are 4.9% and 2.1%.

### 2.1.12 Vitamin D Binding Protein

Vitamin D Binding Protein (VDBP) measurement was conducted in the University of Surrey laboratory, in plasma samples, using the Human Vitamin D BP Immunoassay Quantikine ELISA (R&D Systems Europe, Ltd). Manufacturer’s quoted inter- and intra-assay CVs are 6.2% and 5.9%.

### 2.1.13 Plasma Lipid Profiles

Plasma triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL-C) and non-esterified fatty acid (NEFA) were analysed in-house using the ILAB 650 (Instrumentation Laboratory, Milan, Italy). The ILAB uses an enzymatic colorimetric method incorporating a sequence of step-wise reactions specific to the marker as described below. Low-density lipoprotein cholesterol (LDL-C) was then calculated from total cholesterol, triglyceride and HDL-C concentrations.

#### 2.1.13.1 Calculating LDL Cholesterol

LDL cholesterol was calculated using the Friedewald formula (Friedewald et al. 1972):

\[
LDL = \text{Total cholesterol} - \left( \frac{\text{Triglyceride concentrations}}{5} + \text{HDL concentrations} \right)
\]

#### 2.1.13.2 Principle of colorimetric method per marker:

**Key:**

- 4-AAP = 4-aminoantipyrine,
- TOOS = N-ethyl-N-(2hydroxy-3-sulphopropyl)m-toluidine,
- HDAOS = N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline.

#### 2.1.13.2.1 Triglycerides

Triglycerides – IL Test™ 0018255640 – Instrumentation Laboratory, UK
METHODOLOGY

Triglycerides $\xrightarrow{\text{lipoprotein lipase}}$ glycerol + fatty acids

glycerol + ATP $\xrightarrow{\text{glycerol kinase}}$ glycerol-3-phosphate + ADP

glycerol-3-phosphate + O$_2$ $\xrightarrow{\text{glycerophosphate oxidase}}$ dihydroxyacetone phosphate + H$_2$O$_2$

H$_2$O$_2$ + 4-chlorophenol + 4-AA $\xrightarrow{\text{glycerophosphate oxidase}}$ quinoneimine + H$_2$O$_2$

Production of quinoneimine is proportional to sample triglyceride concentration; quantified via spectrophotometry (primary wavelength absorbance = 510nm, blanking wavelength absorbance = 700nm). Mean intra-assay quality control data: QC1 CV = 3.0%; QC2 CV = 1.7%.

2.1.13.2.2 Total Cholesterol

Total cholesterol – IL Test™ 0018250540 – Instrumentation Laboratory, UK

Cholesterol ester + H$_2$O $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + fatty acids

cholesterol + O$_2$ $\xrightarrow{\text{cholesterol oxidase}}$ cholest-4-en-3-one + H$_2$O$_2$

2H$_2$O$_2$ + 4-AA + phenol $\xrightarrow{\text{Peroxidase}}$ quinoneimine + 4H$_2$O

Production of quinoneimine is proportional to sample total cholesterol concentration; quantified via spectrophotometry (primary wavelength absorbance = 510nm, blanking wavelength absorbance = 700nm). Mean intra-assay quality control data: QC1 CV = 0.0%; QC2 CV = 2.7%.

2.1.13.2.3 HDL Cholesterol

HDL-Cholesterol (two stage reaction) – CH 2655 – Randox Laboratories, UK

1. Clearance of chylomicron, VLDL and LDL-cholesterol

Cholesterol ester $\xrightarrow{\text{cholesterol esterase}}$ cholesterol + fatty acids

cholesterol + O$_2$ $\xrightarrow{\text{cholesterol oxidase}}$ cholestenone + H$_2$O$_2$

2H$_2$O$_2$ $\xrightarrow{\text{Catalase}}$ 2H$_2$O$_2$ + O$_2$
2. Release of HDL-cholesterol by detergents

\[
\text{Cholesterol ester} \xrightarrow{\text{cholesterol esterase}} \text{cholesterol + fatty acids}
\]

\[
\text{cholesterol + } O_2 \xrightarrow{\text{cholesterol oxidase}} \text{cholestenone + } H_2O_2
\]

\[
2H_2O_2 + 4-\text{AA + HDAOS} \xrightarrow{\text{Peroxidase}} \text{quinoneimine + } 4H_2O
\]

Production of quinoneimine is proportional to sample HDL-cholesterol concentration; quantified via spectrophotometry - absorbance = 600nm. Mean intra-assay quality control data: QC1 CV = 0.7%; QC2 CV = 3.1%, QC3 CV = 1.9%

2.1.13.2.4 Non-esterified fatty acid (NEFA)

Non-esterified fatty acid (NEFA) – FA 115 – Randox Laboratories, UK

\[
\text{NEFA+ATP+CoA} \xrightarrow{\text{Acyl CoA Synthetase}} \text{Acyl CoA+AMP+Ppi}
\]

\[
\text{Acyl CoA+O}_2 \xrightarrow{\text{Acyl CoA Oxidase}} 2,3,\text{-trans-Enoyl-CoA+H}_2O_2
\]

\[
2H_2O_2+\text{TOOS+4-AAP} \xrightarrow{\text{Peroxidase}} \text{quinoneimine +4H}_2O
\]

Quinoneimine quantified via spectrophotometry (absorbance = 550nm) and measured against the reagent blank. Mean intra-assay quality control data: QC1 CV = 1.6%; QC2 CV = 1.1%

2.2 The D2-D3 Genetic Study

2.2.1 Sample Collection/Population

Participants recruited onto the D2-D3 Main Study (Chapter 2.1) were also invited to take part in an optional D2-D3 Genetic Study, subject to reading an additional sub-study information sheet (Appendix 33) and signing an additional separate consent form (Appendix 34).
2.2.2 Study Procedures

Participation in the D2-D3 Genetic Study, alongside the D2-D3 Main Study, meant that an additional 5.5ml of blood were taken at each visit to the University. Study procedures remained otherwise identical to those for the D2-D3 Main Study, as described in Chapter 2.1.

The additional blood samples were taken for DNA and RNA extraction. Whole blood (3ml) samples were collected in EDTA tubes for DNA extraction. Blood samples for RNA extraction (2.5ml) were collected in PAXgene Blood RNA Tubes (Becton Dickinson), which contain a reagent that lyses blood cells and immediately stabilises intracellular RNA to preserve the gene expression profile. The order of blood sampling remained the same for participants taking part in the sub study, and the additional two tubes were added to the end of the blood draw with the 3ml EDTA being taken first, followed by the PAXgene tube. The 3ml EDTA tubes were not processed and the whole bloods were transferred directly into a -20°C freezer for 24 hours and then into a -80°C freezer. PAXgene Blood RNA Tubes were inverted ten times, stored upright at 15-25°C for 24 hours, followed by a -20°C freezer for 24 hours and then into a -80°C freezer for long-term storage.

2.2.3 Further Methods

Further methods for the RNA extraction and micro-array analyses are discussed in Chapter 6, and the methods for the DNA extraction and SNP analyses are discussed in Chapter 7.

2.3 Statistical Methods

All statistical analyses were performed with the use of SPSS ® software (version 22.0; SPSS Inc., Chicago, IL). Data were checked for normality using the Kolmogorov-Smirnov test if sample size was >50 or Shapiro-Wilk otherwise. Where data were not normally distributed, non-parametric tests were used. Differences were considered to be significant at p<0.05, unless otherwise stated. Further detail of statistical methods used is described within each study chapter.
Chapter 3 - THE D2-D3 STUDY
3.1 INTRODUCTION

3.1.1 Background

There has been increasing evidence of the beneficial effects of vitamin D in reducing risk from several diseases and all-cause mortality (Garland et al. 2014; Bjelakovic et al. 2011). This has increased awareness of the importance of vitamin D amongst researchers, health professionals, policy makers and even the general public, and highlights the need for evidence-based strategies to address the issue of low vitamin D status in the UK (Pearce and Cheetham 2010; Lanham-New 2010; Spiro & Buttriss 2014; Lanham-New & Wilson 2016). This requirement is particularly relevant and urgent within our multi-cultural society in which there are large numbers of minority groups at a significantly increased risk of vitamin D deficiency (Boucher 2006; Darling et al. 2013a). The issue of poor availability of naturally occurring vitamin D within the UK will make it difficult for the UK population to achieve the recently updated reference nutrient intake (RNI) of 10μg/d set by Public Health England’s Scientific Advisory Committee on Nutrition (SACN 2015). Although taking a vitamin D supplement would improve vitamin D concentrations, relying on supplements would not be an appropriate public health strategy across the population distribution because supplements are only effective in those who actually consume them, and supplementation would be more appropriate under medical-supervision as opposed to the unsupervised majority (Kiely and Cashman 2015). While food fortification offers an alternative and a population-wide solution for improving vitamin D concentrations (Black et al. 2012), research is needed to determine the most effective means of vehicle, optimal concentration and chemical form of vitamin D to maximise the effectiveness of fortification in the UK.

3.1.2 Vitamin D Status and Intakes in UK

In a large UK based study of 7,437 Caucasian men and women, during the winter/spring seasons 15.5% were deficient in vitamin D (25OHD concentrations <25 nmol/L) (Hyppönen and Power 2007). In the most recent National Diet and Nutrition Survey (NDNS) collected in 2011/12, 24% of men and 21.7% of women aged 19-64 years of 3,450 adults were deficient (Bates et al. 2014). The mean 25OHD concentrations of a sub-set of these men and women (n1,769) were 43.5nmol/L and 47.3nmol/L respectively (Bates et al. 2014).
3.1.3 Vitamin D ‘crisis’ in UK South Asians

Ethnic minority groups, in particular South Asian women, in the UK are at a particular risk of vitamin D deficiency due to lifestyle (sun exposure habits, traditional clothing), diet (vegan and vegetarian groups) and darker skin tones (affecting vitamin D₃ synthesis). The D-FINES study was the first longitudinal study investigating vitamin D concentrations in South Asian women. The study showed that the South Asian women had almost year-round vitamin D deficiency (<25 nmol/L), and showed very little seasonal variation, unlike the Caucasian women who had 25OHD concentrations >50 nmol/L in the summer months but decreased to <50 nmol/L in the winter months (Macdonald et al. 2011, Darling et al. 2013a). Although some of the increased risk of vitamin D deficiency in South Asians can be explained by lifestyle and diet, there is also some evidence for genetic differences. In particular, studies have shown different allele frequencies in polymorphisms in key genes and expression of the enzymes known to affect vitamin D metabolism between Asian Indian and Caucasian populations (Bid et al. 2005).

3.1.4 Dietary Vitamin D Recommendations

Prior to the proposed update to the UK dietary recommendations for vitamin D (SACN 2015), there was only a recommendation set for vitamin D intakes in the UK for infants (<5yrs), the elderly (>65 yrs) and population groups at risk of low sun exposure (Department of Health 1991; Department of Health 1998; SACN 2007), as it was previously thought that sufficient 25OHD concentrations were achieved in the summer months from sun exposure to last throughout the winter, however this is now known not to be the case (Macdonald et al. 2011; Darling et al. 2013a). The seasonal fluctuations that are shown in 25OHD concentrations of Caucasian women have even been suggested to be detrimental to bone (Darling et al. 2014; Darling et al. 2015). Solutions for increasing vitamin D intakes are therefore needed in the UK, for both South Asian and Caucasian women, in order to achieve the new dietary intakes recommended and to improve and maintain 25OHD concentrations.

3.1.5 Limited Availability of Vitamin D in the UK

Natural dietary sources of vitamin D in the UK are very limited and few foods are fortified, as discussed in Chapter 1.7. The main natural food source of vitamin D in the UK is oily fish, but only 23% of the UK adult population are oily fish consumers (Bates et al. 2014). Dietary intakes of vitamin D are consistently low in comparison to the newly proposed RNI of 10μg/d, at intakes of around 2.0-2.5 μg/d (80-100 IU/d) in Caucasian women and 1.25-1.6 μg/d (50-65IU/d) in South Asian women.
(Macdonald et al. 2011). The National Diet and Nutrition Survey (NDNS) data collected in 2011/12 show mean dietary intakes of vitamin D of 3.9 and 3.4µg/d in men and women respectively (Bates et al. 2014), with approximately 0.8 µg of this, for both the men and women, coming from fortified foods. Vitamin D supplement use in this survey was 25% in men and 24% in women (Bates et al. 2014), and although this is higher than in the previous NDNS (Henderson et al. 2003), it still only represents a quarter of the UK adult population. The ability to make vitamin D3 from sunlight exposure (as discussed in Chapters 1.2.2.1 and 1.8.2.3) is also limited to between the months of April-October in the UK, thereby eliminating a potential source of vitamin D for half of the year.

3.1.6 Controversy in the Effectiveness of Vitamin D2 and Vitamin D3

Vitamin D is the generic term for two molecules; vitamin D2 and vitamin D3 as introduced in Chapter 1.2.1. It has been assumed for some time, largely on the strength of studies from the 1930s (Park, 1940), that D2 and D3 were equally effective in humans. However, more recent studies since the 1980s have shown mixed results, as discussed in Chapter 1.9. There are ten intervention trials comparing supplementation with vitamin D2 vs. vitamin D3 in the past 20 years that have shown that vitamin D3 is superior to D2 in raising 25OHD concentrations (Tjellnesen et al. 1986; Trang et al. 1998; Armas et al. 2004; Romagnoli et al. 2008; Glendenning et al. 2009; Binkley et al. 2011; Heaney et al. 2011; Logan et al. 2013; Lehmann et al. 2013; Oliveri et al. 2015), however three intervention trials have shown that vitamin D2 and D3 are equally effective (Holick et al. 2008, Biacuzzo et al. 2010; Fisk et al. 2012). Therefore, as discussed in Chapter 1.9, there is ongoing controversy as to the effectiveness of vitamin D2 and D3 in raising and maintaining 25OHD concentrations in humans (Houghton & Vieth 2006; Lanham-New et al. 2010; Tripkovic et al. 2012).

Houghton and Vieth (2006) reviewed the many biologically plausible mechanisms that could contribute to the greater capacity of vitamin D3 over D2 to maintain higher 25OHD concentrations over time. As discussed in Chapter 1.2.3.4, there a several stages within the metabolism of both forms at which vitamin D3 may have an advantage. The vitamin D binding protein (VDBP) and 25-hydroxylase both have a higher binding affinity for vitamin D3 and 25OHD3 than vitamin D2 and 25OHD2 (Holmerg et al. 1986; Cheng et al. 2003; Cheng et al. 2004). This suggests that vitamin D3 would be taken up and transported to the liver faster than vitamin D2, and also hydroxylated to 25OHD3 faster than vitamin D2, as well as reaching the kidney for conversion to the active form (1,25(OH)2D3) faster than vitamin D2 (Houghton & Vieth 2006). The degradation of vitamin D3 also differs slightly to that of vitamin D2, and gives vitamin D3 the potential to remain biologically active for a greater period.
3.1.7 Stability and Bioavailability of Vitamin D2 and D3 in Fortified Foods

While food fortification offers a population-wide solution for improving vitamin D concentrations (Black et al. 2012), research is needed to determine the stability and bioavailability of vitamin D2 and vitamin D3 in a wider range of foods and vehicles than have been confirmed so far, particularly among different population groups (Grossman & Tangpricha 2010). The stability of vitamin D2 has been suggested to be less stable than vitamin D3, particularly on exposure to high temperature (Grady & Thacker 1980), which highlights the importance of stability data being presented alongside results from any vitamin D2 vs. D3 comparative studies. As discussed in Chapter 1.7.3, vitamin D is lipid soluble and it has been speculated that vitamin D may require other lipids to not only stimulate the release of bile acids in the duodenum to facilitate lipid absorption (Basu et al. 2003) and therefore foods with a higher fat content may lead to more bioavailable vitamin D than foods within lower or no fat content.

3.1.8 Justification

As highlighted in the meta-analysis by Tripkovic and colleagues (2012) and many review papers (Houghton & Vieth 2006; Lanham-New et al. 2010; Tripkovic et al. 2012), to date there have been few sufficiently powered studies comparing vitamin D2 vs D3 in well designed, randomised controlled trials (RCTs). Specifically lacking are studies including lower and more achievable daily doses, without the need for supplementation. Since the 2012 meta-analysis discussed in Chapter 1.9, there have been four further RCTs comparing the two forms of vitamin D in more achievable daily doses (Fisk et al. 2012; Logan et al. 2013; Lehmann et al. 2013; Oliveri et al. 2015), yet study numbers have still been low and results remain inconsistent. Therefore, there remains a need for a large randomised controlled trial comparing an achievable or recommended daily dose of vitamin D2 vs. D3 to try and resolve the debate once and for all.

In 2010, the IOM Report set updated dietary recommendations for vitamin D of 15 μg/d in the US, with several European/European Countries Guidelines being updated to 10-15 μg/d too (Chapter 1.5.2.1). In the UK, the guidelines had not been updated since 1998, until the release of the SACN report in draft form in July 2015. The proposed RNI for vitamin D in the UK is 10 μg/d (SACN 2015). Across the UK, Europe and also North America, solutions for achieving a daily intake of 10-15 μg/d are needed and food-based approaches, such as food fortification, is one of these potential solutions (Kiely & Cashman 2014). However, more evidence is needed on the efficacy, dose and safety of
vitamin D food fortification, as well as determining whether one form of vitamin D would be most suitable and whether vehicle of fortification impacts on the bioavailability of vitamin D.

With the South Asian population in the UK making-up 7.5% of the total UK population (Office for National Statistics 2012), and vitamin D deficiency as high as 64.5% in the winter-time in South Asian women (MacDonald et al. 2011), it is vital to determine effective solutions for improving vitamin D status. Many South Asians also follow a vegetarian diet, within their Hindu religion, which would limit their dietary sources of vitamin D. Despite this urgent need for attention in the South Asian populations, the majority of UK-based fortification and supplementation trials to date have been conducted in Caucasian populations. There are also no studies comparing the efficacy of vitamin D₂ vs. D₃ within ethnic minority groups in the UK, nor elsewhere. This limits the ability for policy makers and government organisations to consider such at risk groups when implementing strategies for improving vitamin D status, as there is a lack of evidence to consider.

This study will provide essential information for:

i) policy makers and the UK food industry; on the comparative effectiveness of the two forms of vitamin D in raising 25OHD concentrations in the UK populations with known deficiencies (South Asian) and insufficiencies (Caucasian), the response to the daily dose of vitamin D recommended by the IOM in the USA, and the feasibility of delivering vitamin D via different vehicles/foods fortified;

ii) the scientific community; with detailed data on the mechanism of action of any such differences in vitamin D₂ vs. D₃ in Caucasian and South Asians;

iii) the general population; with greater knowledge of vitamin D and examples of how to improve vitamin D intakes through food fortification, which would be particularly important within South Asian populations and also vegetarian and vegan groups.

3.1.9 Aims and Hypothesis

**Aim 1** - To compare the efficiency of 15 µg/d [600 IU/d] of vitamin D₂ vs. vitamin D₃, delivered via fortification of food products, in raising total serum 25OHD concentrations in South Asian and Caucasian women independently.

**Hypothesis** – Vitamin D₃, compared to vitamin D₂, would be more effective at raising total serum 25OHD concentrations in both the South Asian and Caucasian women
**Aim 2** - To compare the effects of vehicle for fortification (i.e. a solid vs. liquid food) with vitamin D$_2$ vs. vitamin D$_3$ in raising serum 25OHD concentrations, in South Asian and Caucasian women independently.

**Hypothesis** - The solid food (biscuit) would be a more suitable food fortification vehicle for vitamin D food fortification, compared to the liquid food (orange juice), in both the South Asian and Caucasian women.

**Aim 3** – To assess whether 15 µg/d (600 IU/d) vitamin D$_2$ and/or vitamin D$_3$ is effective in raising wintertime serum 25OHD concentrations above ‘deficiency’ (25 nmol/L) and ‘insufficiency’ (50 nmol/L) thresholds over 12-weeks, in both South Asian and Caucasian women independently.

**Hypothesis** – A dose of 15 µg/d (600IU/d) of either vitamin D$_2$ or D$_3$ for 12 weeks would bring those with 25OHD concentrations in the ‘deficient’ (<25nmol/L) range at baseline into the ‘insufficient’ range (25-50nmol/l), and a proportion of those with 25OHD concentrations in the ‘insufficient’ range at baseline into the ‘sufficient’ (50-75nmol/L) range in both the South Asian and Caucasian women.

**Aim 4** – To compare the effects of 15 µg/d (600 IU/d) vitamin D$_2$ vs. vitamin D$_3$ on vitamin D metabolites (25OHD$_2$, 25OHD$_3$, and vitamin D binding protein (VDBP) concentrations), in order to determine possible mechanisms for any differences that may be shown in Aim 1.

**Hypothesis** – In the vitamin D$_2$ intervention, 25OHD$_2$ concentrations would increase and 25OHD$_3$ concentrations would decrease, and in the vitamin D$_3$ intervention 25OHD$_3$ concentrations would increase, in both the South Asian and Caucasian women. VDBP concentrations would increase in both the vitamin D$_2$ and vitamin D$_3$ interventions, and more so in the vitamin D$_3$.

**Aim 5** – To compare the changes in 25OHD, 25OHD$_2$ and 25OHD$_3$ concentrations in those on the vitamin D$_2$ vs. vitamin D$_3$ interventions, 4-weeks after the 12-week supplementation trial ended.

**Hypothesis** – Total 25OHD and 25OHD$_2$ concentrations would decrease greater in the vitamin D$_2$ intervention, than total 25OHD and 25OHD$_3$ concentrations within the vitamin D$_3$ intervention in the four weeks after the intervention.
3.2 METHODS

3.2.1 The D2-D3 Study

The D2-D3 study design, ethical approval, recruitment, randomisation and protocols have been discussed previously in detail in Chapter 2, so are not repeated here. In summary, healthy South Asian and Caucasian women aged 20-65 years were randomised to receive placebo, 15 μg/d vitamin D$_2$ or 15 μg/d vitamin D$_3$, delivered via fortified foods, for a 12-week period and attended three study visits for data collection; week 0, week 6 and week 12. A small sub-set of participants were invited to attend a fourth study visit at week 16, four weeks after the intervention had ended.

3.2.2 Relevant Data

The outcome variables of relevance to this chapter include participant anthropometrics, dietary intakes and sunlight exposure data, total 25OHD concentrations (including 25OHD$_2$ and 25OHD$_3$) and vitamin D binding protein concentrations (VDBP), across all three visits. Anthropometrics, vitamin D concentrations and vitamin D metabolite concentrations from the sub-set of participants who took part in a fourth visit are also used within these analyses.

3.2.3 Statistical Analysis

All statistical analyses were performed with the use of SPSS® software (version 22.0; SPSS Inc., Chicago, IL). Differences were considered to be significant at $p<0.05$. Data were checked for normality using the Kolmogorov-Smirnov test if sample size was $>50$ or Shapiro-Wilkinson otherwise. Both the anthropometric measurement data, and the 25OHD data were not normally distributed, and log transforming the data did not normalise them, therefore non-parametric tests were used throughout these analyses.

Comparisons between the two ethnic groups at baseline were conducted using Mann-Whitney U tests. Within each ethnic group, comparisons between intervention groups at each visit independently (including baseline), and between total change and percentage change in outcome variables, were performed by Kruskal-Wallis tests, and where significant was followed by Mann-Whitney U tests with Bonferroni adjustment to the $p$-value for significance. Comparisons between measures across three (or four) different time points within each intervention group were performed...
using a Friedman test, and where significance was found this was followed by Wilcoxon Signed Rank tests with Bonferroni adjustment to the p-value for significance.

Results are presented as mean values and standard deviations (SD) in the text and tables, and as mean values and standard error of the mean (SEM) in graphs.

Intention-to-treat (ITT) analyses have also been completed on this data set with the same results shown, although this data is not presented within this chapter.

3.3 RESULTS

3.3.1 Recruited Participants

A total of 335 women were recruited onto the D2-D3 Study, of which 90 were South Asian and 245 were Caucasian, and were randomised to one of the five intervention groups; placebo, D2 juice, D2 biscuit, D3 juice or D3 biscuit.

3.3.2 Compliance and Attrition

3.3.2.1 Compliance and Drop Outs

Compliance was assessed using methods described in Chapter 2.1.9.4. Over the 12-week intervention, in the participants that completed the study, participants consumed 94% of the products allocated to them.

Throughout the study there was a total of 44 drop-outs, equating to a 13% drop-out rate. Of these dropouts, 27 were South Asian (30% drop-out rate) and 17 were Caucasian (5% drop-out rate). A total of 291 participants therefore successfully completed the study; 63 South Asians and 228 Caucasians, as shown in Figure 3-1. The reason for drop-outs are also shown in Figure 3-1.
Figure 3-1 CONSORT flow-diagram indicating participant allocation and attrition through the D2-D3 Study
3.3.3 Participant Anthropometrics and Biochemistry

3.3.3.1 Ethnic Group Anthropometrics

A total of 335 women were recruited onto the D2-D3 Study, of which 90 were South Asian and 245 were Caucasian. The baseline anthropometric characteristics and 25OHD concentrations for the South Asian and Caucasian women are shown in Table 3.1. The two ethnic groups had significantly different anthropometric measurements and 25OHD concentrations. In the South Asian and Caucasian cohorts, mean age was 37.07 ±10.32 and 45.71 ±12.24 years, respectively (p<0.001), BMI was 25.31 ±4.37 and 23.57 ±3.46 kg/m², respectively (p<0.001), and 25OHD concentration was 27.55 ±22.25 and 60.28 ±26.54 nmol/L, respectively (p<0.001).

Table 3.1 Descriptive data of anthropometric values and 25OHD concentrations (mean ±SD), separated by ethnic group

<table>
<thead>
<tr>
<th></th>
<th>South Asian</th>
<th>Caucasian</th>
<th>Mann-W1 p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
</tr>
<tr>
<td>Age (years)</td>
<td>90</td>
<td>37.07 ±10.32</td>
<td>245</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>90</td>
<td>25.31 ±4.37</td>
<td>245</td>
</tr>
<tr>
<td>Bodyfat (%)</td>
<td>90</td>
<td>32.64 ±6.96</td>
<td>245</td>
</tr>
<tr>
<td>Waist:Hip Ratio</td>
<td>90</td>
<td>0.84 ±0.08</td>
<td>245</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>89</td>
<td>112.96 ±13.31</td>
<td>245</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>89</td>
<td>76.43 ±9.81</td>
<td>245</td>
</tr>
<tr>
<td>Total 25OHD (nmol/L)</td>
<td>90</td>
<td>27.55 ±22.25</td>
<td>245</td>
</tr>
</tbody>
</table>

Mann-Whitney U-Test, BMI= body mass index, BP = blood pressure

The participants were randomised, as per the procedures described in Chapter 2.1.6, to one of five intervention groups: placebo, D₂ juice, D₂ biscuit, D₃ juice or D₃ biscuit.

3.3.3.2 Intervention Groups Anthropometrics

Participant characteristics by treatment group, with the two ethnic groups independently, are shown in Table 3.2. As shown in this table, there were no significant differences between the five treatment groups for any of the anthropometric measurements within the South Asian (p>0.351 in all cases) or the Caucasian cohorts (p>0.305 in all cases). However, when comparing ethnic groups (South Asian vs. Caucasian) within each treatment group, there were significant differences in all of the measures except hip circumference and diastolic blood pressure. The significant p-values are shown in blue in Table 3.2.
<table>
<thead>
<tr>
<th></th>
<th>Age (yrs)</th>
<th>Height (m)</th>
<th>Weight (kg)</th>
<th>BMI (kg/m²)</th>
<th>Waist Circ (cm)</th>
<th>Hip Circ (cm)</th>
<th>Waist:Hip Ratio</th>
<th>Body Fat (%)</th>
<th>Systolic BP (mmHg)</th>
<th>Diastolic BP (mmHg)</th>
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</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
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<td>South Asian n17</td>
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<td></td>
<td>38.9</td>
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<td>±10.1</td>
<td>±11.7</td>
<td>±11.1</td>
<td>±10.6</td>
<td>±10.9</td>
<td>±5.8</td>
<td>±0.07</td>
<td>±6.8</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>±9.5</td>
<td>±10.1</td>
<td>±11.7</td>
<td>±11.1</td>
<td>±10.6</td>
<td>±10.9</td>
<td>±5.8</td>
<td>±0.07</td>
<td>±6.8</td>
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<tr>
<td>Caucasian n47</td>
<td>Mean</td>
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<td>45.8</td>
<td>±11.7</td>
<td>±10.1</td>
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<td>±9.3</td>
<td>±6.7</td>
<td>±0.07</td>
<td>±5.6</td>
<td>±14.8</td>
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<tr>
<td></td>
<td>SD</td>
<td>±11.7</td>
<td>±10.1</td>
<td>±9.1</td>
<td>±9.3</td>
<td>±6.7</td>
<td>±0.07</td>
<td>±5.6</td>
<td>±14.8</td>
<td>±9.3</td>
</tr>
<tr>
<td><strong>p-value</strong></td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.014</td>
<td>0.034</td>
<td>0.030</td>
<td>0.686</td>
<td>0.002</td>
<td>0.007</td>
<td>0.011</td>
<td>0.218</td>
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<tr>
<td><strong>D2 Juice</strong></td>
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<td>South Asian n18</td>
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<tr>
<td></td>
<td>37.5</td>
<td>±10.1</td>
<td>±7.9</td>
<td>±3.5</td>
<td>±10.9</td>
<td>±5.8</td>
<td>±0.09</td>
<td>±4.3</td>
<td>±13.3</td>
<td>±9.9</td>
</tr>
<tr>
<td></td>
<td>SD</td>
<td>±10.1</td>
<td>±7.9</td>
<td>±3.5</td>
<td>±10.9</td>
<td>±5.8</td>
<td>±0.09</td>
<td>±4.3</td>
<td>±13.3</td>
<td>±9.9</td>
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<tr>
<td>Caucasian n49</td>
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<td></td>
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<tr>
<td></td>
<td>46.3</td>
<td>±11.0</td>
<td>±8.5</td>
<td>±3.3</td>
<td>±9.1</td>
<td>±6.7</td>
<td>±0.07</td>
<td>±5.6</td>
<td>±14.8</td>
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<td>SD</td>
<td>±11.0</td>
<td>±8.5</td>
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<td>±6.7</td>
<td>±0.07</td>
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<tr>
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<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<td>±9.6</td>
<td>±7.6</td>
<td>±0.07</td>
<td>±6.5</td>
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<td>0.509</td>
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<tr>
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<tr>
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<td>±8.4</td>
<td>±0.09</td>
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<td>±4.5</td>
<td>±12.9</td>
<td>±8.4</td>
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<td>±0.07</td>
<td>±6.8</td>
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<td>±9.0</td>
</tr>
<tr>
<td></td>
<td>SD</td>
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<td>±11.1</td>
<td>±3.4</td>
<td>±10.8</td>
<td>±7.4</td>
<td>±0.07</td>
<td>±6.8</td>
<td>±15.3</td>
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</tr>
<tr>
<td><strong>p-value</strong></td>
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<td>0.851</td>
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<td>0.786</td>
<td>0.005</td>
<td>0.113</td>
<td>0.042</td>
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<td><strong>Between Treatment Groups</strong></td>
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</tr>
<tr>
<td>South Asian</td>
<td>p-valuea</td>
<td>0.908</td>
<td>0.351</td>
<td>0.860</td>
<td>0.936</td>
<td>0.993</td>
<td>0.619</td>
<td>0.793</td>
<td>0.918</td>
<td>0.643</td>
</tr>
<tr>
<td>Caucasian</td>
<td>p-valueb</td>
<td>0.998</td>
<td>0.570</td>
<td>0.481</td>
<td>0.712</td>
<td>0.910</td>
<td>0.305</td>
<td>0.430</td>
<td>0.982</td>
<td>0.797</td>
</tr>
</tbody>
</table>

SD = standard deviation, a Mann-Whitney U Test between South Asian and Caucasian, b Kruskal-Wallis Test, between intervention groups, blue highlights significant p-values <0.005
3.3.3.3 Participant Biochemistry

As shown in Table 3.3, both the South Asian and Caucasian cohort had mean corrected calcium, albumin, thyroid stimulating hormone, electrolytes (sodium, potassium and creatinine), urea and liver function test (ALP and ALT) results within healthy reference ranges.

Table 3.3 Participant biochemistry, split by ethnicity (mean ± SD)

<table>
<thead>
<tr>
<th></th>
<th>Reference Range</th>
<th>South Asian</th>
<th></th>
<th></th>
<th></th>
<th>Caucasian</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>Mean</td>
<td>±SD</td>
<td>n</td>
<td>Mean</td>
<td>±SD</td>
<td></td>
</tr>
<tr>
<td>Corrected Calcium</td>
<td>2.10-2.55 mmol/L</td>
<td>90</td>
<td>2.31</td>
<td>±0.07</td>
<td>245</td>
<td>2.33</td>
<td>±0.09</td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>35-50 g/L</td>
<td>90</td>
<td>43.83</td>
<td>±2.00</td>
<td>245</td>
<td>43.81</td>
<td>±1.92</td>
<td></td>
</tr>
<tr>
<td>TSH</td>
<td>0.4-4.5 miU/L</td>
<td>69</td>
<td>1.99</td>
<td>±1.19</td>
<td>236</td>
<td>2.54</td>
<td>±1.96</td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>3.6-5.0 mmol/L</td>
<td>69</td>
<td>4.18</td>
<td>±0.24</td>
<td>235</td>
<td>4.22</td>
<td>±0.33</td>
<td></td>
</tr>
<tr>
<td>Sodium</td>
<td>137-145 mmol/L</td>
<td>69</td>
<td>139.38</td>
<td>±1.28</td>
<td>236</td>
<td>140.02</td>
<td>±1.97</td>
<td></td>
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<tr>
<td>Creatinine</td>
<td>46-92 μmol/L</td>
<td>69</td>
<td>55.39</td>
<td>±7.04</td>
<td>236</td>
<td>63.18</td>
<td>±8.49</td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>2.5-7.5 mmol/L</td>
<td>69</td>
<td>4.36</td>
<td>±1.12</td>
<td>236</td>
<td>4.69</td>
<td>±1.06</td>
<td></td>
</tr>
<tr>
<td>ALP</td>
<td>30-126 U/L</td>
<td>69</td>
<td>64.52</td>
<td>±16.92</td>
<td>236</td>
<td>59.80</td>
<td>±17.62</td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>9-52 U/L</td>
<td>64</td>
<td>14.03</td>
<td>±4.63</td>
<td>229</td>
<td>15.17</td>
<td>±6.50</td>
<td></td>
</tr>
<tr>
<td>Total Bilirubin</td>
<td>3-22 μmol/L</td>
<td>69</td>
<td>8.48</td>
<td>±4.12</td>
<td>236</td>
<td>10.72</td>
<td>±4.91</td>
<td></td>
</tr>
</tbody>
</table>

Key: ^1 Surrey Pathology Services, NHS Pathology (2014), TSH thyroid stimulating hormone, ALP alkaline phosphatase, ALT alanine aminotransferase

3.3.4 Exogenous sources of vitamin D

3.3.4.1 Dietary Intakes Pre-Intervention

Diet diaries were returned and of sufficient detail for analysis from 262 participants (78% response rate); 33 South Asian (37% response rate) and 229 Caucasian (94% response rate).

The mean dietary intakes of the South Asian and Caucasian women are shown in Table 3.4. The mean intake of dietary vitamin D was 2.24 ±2.06 µg/d and 2.77 ±2.38 µg/d respectively (p=0.075), and calcium intake was 703 ±211 mg/d and 870 ±261 mg/d respectively (p=0.001).

Comparisons of intakes between intervention groups, also shown in Table 3.4, showed calcium intakes were significantly different, although post-hoc analyses (with bonferroni adjustment) showed no significant differences.
Table 3.4 Dietary intakes in the South Asian and Caucasian women

<table>
<thead>
<tr>
<th></th>
<th>South Asian n33</th>
<th>Caucasian n229</th>
<th>Between Ethnic Groups p-value a</th>
<th>Between Intervention Groups p-value b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kCal) (KJ)</td>
<td>1827.5 ±434.5</td>
<td>1909.3 ±447.1</td>
<td>0.421</td>
<td>0.847</td>
</tr>
<tr>
<td></td>
<td>7675.3 ±1816.6</td>
<td>8011.9 ±1871.5</td>
<td>0.424</td>
<td>0.870</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>68.07 ±18.21</td>
<td>73.46 ±18.07</td>
<td>0.162</td>
<td>0.230</td>
</tr>
<tr>
<td></td>
<td>206.81 ±43.94</td>
<td>204.33 ±52.75</td>
<td>0.504</td>
<td>0.729</td>
</tr>
<tr>
<td>Carbohydrate (g)</td>
<td>82.97 ±28.09</td>
<td>92.55 ±32.91</td>
<td>0.167</td>
<td>0.246</td>
</tr>
<tr>
<td>Total Sugar (g)</td>
<td>79.41 ±27.49</td>
<td>78.48 ±26.1</td>
<td>0.762</td>
<td>0.930</td>
</tr>
<tr>
<td>Total Fat (g)</td>
<td>11.71 ±4.09</td>
<td>14.44 ±4.7</td>
<td>0.003</td>
<td>0.071</td>
</tr>
<tr>
<td>NSP (g)</td>
<td>0.58 ±2.63</td>
<td>17.3 ±86.34</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Alcohol (g)</td>
<td>2.24 ±2.06</td>
<td>2.77 ±2.38</td>
<td>0.075</td>
<td>0.176</td>
</tr>
<tr>
<td>Calcium (mg)</td>
<td>702.68 ±211.17</td>
<td>870.14 ±260.95</td>
<td>0.001</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Key: % EI = percentage of energy intake, NSP=non starch polysaccharide, a Mann-Whitney U Test, b Kruskal-Wallis test between South Asian (SA) placebo, SA D2, SA D3, Caucasian (C) placebo, C D2 and C D3.

3.3.4.2 UVB Exposure

Participants’ UV exposure for the duration of the trial was minimal, with a mean exposure of 0.035 ±0.039 SED/week pre-intervention and 0.086 ±0.137 SED/week post-intervention for the cohort. 1 SED fortnightly, and therefore 0.5 SED/week, is enough to maintain 25OHD concentrations (Bogh et al. 2012), and the participants in this study only reached 7% of this pre-intervention and 17% post-intervention.

3.3.5 Baseline 25OHD Concentrations

At baseline the South Asian participants had significantly lower 25OHD concentrations than the Caucasian women (27.6 ±22.2nmol/l and 60.3 ±26.5nmol/l respectively, p<0.001), and this was a consistent finding when comparing baseline 25OHD concentrations between ethnic groups within each intervention group, as shown in Table 3.5. Within each ethnic group, 25OHD concentrations were not significantly different between intervention groups at baseline (Table 3.5).

The proportion of South Asian and Caucasian participants who were ‘deficient’ (<25 nmol/L) was 62% and 4%, ‘insufficient’ (<50 nmol/L) was 83% and 37%, ‘sufficient’ (<75 nmol/L) was 96% and 76% and ‘optimal’ (>75 nmol/L) was 4% and 24%, respectively (Figure 3-2).
Figure 3-2 Percentage of South Asian and Caucasian participants with 25OHD concentrations within cut-offs. Percentage of participants, within each ethnic group, to have deficient (<25nmol/L), insufficient (25-50 nmol/L), sufficient (50-75 nmol/L) and optimal (>75 nmol/L) 25OHD concentrations.

3.3.6 Change in 25OHD Concentrations: Comparison Between Vehicles

Total 25OHD concentrations at each visit, and total and percentage change in 25OHD concentrations from week 0 to week 12, within each intervention group, stratified by ethnicity are shown in Table 3.5.

3.3.6.1 Food Vehicle Comparison

In both ethnic groups there was no significant difference in total change in 25OHD, between the D$_2$ juice and D$_2$ biscuit intervention (SA: 18.80 and 17.54 nmol/l respectively, p=1.000; Cauc: 11.70 and 13.33 nmol/l respectively, p=0.995), and between the D$_3$ juice and D$_3$ biscuit intervention (SA: 32.15 and 34.78 nmol/l respectively, p=0.997; Cauc: 30.53 and 29.82 nmol/L respectively, p=1.000).

The biscuit and juice groups were therefore combined forming three groups, within each ethnic group, for all further analyses; placebo, vitamin D$_2$ (D$_2$ juice and D$_2$ biscuit) and vitamin D$_3$ (D$_3$ juice and D$_3$ biscuit).
Table 3.5 Serum 25OHD concentrations (mean ±SD) across all three visits, split by ethnicity and the five intervention groups

<table>
<thead>
<tr>
<th>Total serum 25OHD (nmol/l)</th>
<th>n</th>
<th>Week 0 (V1) Mean ±SD</th>
<th>n</th>
<th>Week 6 (V2) Mean ±SD</th>
<th>n</th>
<th>Week 12 (V3) Mean ±SD</th>
<th>Over Time p-value</th>
<th>Δ change (V1-V3) Mean ±SD</th>
<th>% change (V1-V3) Mean ±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>17</td>
<td>30.77±27.21</td>
<td>15</td>
<td>18.43±9.48</td>
<td>14</td>
<td>19.53±7.87</td>
<td>0.023</td>
<td>-5.09±10.00</td>
<td>-9.64±36.48</td>
</tr>
<tr>
<td>Caucasian Mean ± SD</td>
<td>48</td>
<td>58.80±23.10</td>
<td>46</td>
<td>49.61±19.74</td>
<td>45</td>
<td>43.87±18.10</td>
<td>&lt;0.001</td>
<td>-15.28±11.26</td>
<td>-24.94±13.24</td>
</tr>
<tr>
<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
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<tr>
<td><strong>D&lt;sub&gt;2&lt;/sub&gt; Juice</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>18</td>
<td>29.46±24.14</td>
<td>15</td>
<td>44.68±15.38</td>
<td>13</td>
<td>48.39±16.54</td>
<td>0.009</td>
<td>18.80±19.57</td>
<td>121.88±117.76</td>
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<tr>
<td>Caucasian Mean ± SD</td>
<td>49</td>
<td>60.30±27.55</td>
<td>49</td>
<td>71.73±18.24</td>
<td>47</td>
<td>72.13±18.61</td>
<td>&lt;0.001</td>
<td>11.70±19.35</td>
<td>34.01±48.42</td>
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<tr>
<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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<tr>
<td><strong>D&lt;sub&gt;2&lt;/sub&gt; Biscuit</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>17</td>
<td>30.46±22.57</td>
<td>15</td>
<td>46.39±18.07</td>
<td>14</td>
<td>52.06±13.20</td>
<td>0.008</td>
<td>17.54±15.67</td>
<td>102.76±96.79</td>
</tr>
<tr>
<td>Caucasian Mean ± SD</td>
<td>49</td>
<td>61.79±31.47</td>
<td>44</td>
<td>72.35±24.00</td>
<td>44</td>
<td>75.27±22.88</td>
<td>&lt;0.001</td>
<td>13.33±19.78</td>
<td>43.52±63.95</td>
</tr>
<tr>
<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
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<td></td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;3&lt;/sub&gt; Juice</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>19</td>
<td>27.34±21.94</td>
<td>12</td>
<td>58.12±13.85</td>
<td>11</td>
<td>65.45±14.72</td>
<td>0.014</td>
<td>32.15±27.11</td>
<td>239.49±244.57</td>
</tr>
<tr>
<td>Caucasian Mean ± SD</td>
<td>51</td>
<td>57.31±23.31</td>
<td>50</td>
<td>83.72±22.18</td>
<td>48</td>
<td>88.15±22.90</td>
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<td>30.53±22.82</td>
<td>75.61±100.50</td>
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<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;3&lt;/sub&gt; Biscuit</strong></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>19</td>
<td>20.47±15.19</td>
<td>12</td>
<td>47.73±12.92</td>
<td>11</td>
<td>56.28±18.53</td>
<td>&lt;0.001</td>
<td>34.78±20.43</td>
<td>245.76±182.60</td>
</tr>
<tr>
<td>Caucasian Mean ± SD</td>
<td>48</td>
<td>63.38±27.10</td>
<td>47</td>
<td>87.60±22.93</td>
<td>44</td>
<td>93.37±24.91</td>
<td>&lt;0.001</td>
<td>29.82±21.84</td>
<td>67.43±64.70</td>
</tr>
<tr>
<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>&lt;0.001</td>
<td></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Between Intervention</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Groups (p-value)&lt;sup&gt;b&lt;/sup&gt;</td>
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<td></td>
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</tr>
<tr>
<td>South Asian Mean ± SD</td>
<td>0.617</td>
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<td></td>
<td>&lt;0.001</td>
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<td>&lt;0.001</td>
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</tr>
<tr>
<td>Caucasian Mean ± SD</td>
<td>0.811</td>
<td></td>
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<td></td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SD = standard deviation, Δ change (V1-V3) = total change in 25OHD from V1 to V3, % change (V1-V3) = percentage change in 25OHD from V1 to V3, <sup>a</sup>Mann-Whitney U Test between South Asian and Caucasian, <sup>b</sup>Kruskal-Wallis Test, between intervention groups, M = mean, <sup>c</sup>Friedman Test (significant at p<0.05) with post-hoc Wilcoxon Signed Rank Test (sig adjusted to p-value <0.017): <sup>1</sup>significantly different from week 0 in post-hoc analyses, <sup>2</sup>significantly different from week 6 in post-hoc analyses.
3.3.8 Change in 25OHD Concentrations: Juice and Biscuit Groups Combined

With the juice and biscuit groups combined to form three intervention arms, the total 25OHD concentrations at each visit and the change in 25OHD from week 0 to week 12 are shown in Table 3.6, and presented graphically in Figure 3-3.

![Figure 3-3](image-url)

**Figure 3-3 Total 25OHD concentrations of both South Asian and Caucasian participants, in the three intervention groups, over the three study visits.**

Mean [±SEM] total 25OHD concentrations at baseline (week 0), week 6 and week 12 for the South Asian (SA) placebo, Caucasian (Cauc) placebo, SA Vitamin D2, Cauc Vitamin D2, SA Vitamin D3 and Cauc Vitamin D3 intervention groups. **significant difference over time (p<0.001), *significant difference over time (p<0.03)**
### Table 3.6 Serum 25OHD concentrations of the South Asian and Caucasian women in the three intervention groups, at each of the three visits

<table>
<thead>
<tr>
<th>Total serum 25OHD (nmol/l)</th>
<th>Week 0 (V1)</th>
<th>Week 6 (V2)</th>
<th>Week 12 (V3)</th>
<th>Between Visits</th>
<th>Δ change (V1-V3)</th>
<th>% change (V1-V3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>17</td>
<td>30.77±27.21</td>
<td>15</td>
<td>18.43±9.48</td>
<td>14</td>
<td>19.53±7.87</td>
</tr>
<tr>
<td>Caucasian</td>
<td>48</td>
<td>58.80±23.10</td>
<td>46</td>
<td>49.61±19.74</td>
<td>45</td>
<td>43.87±18.10</td>
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<tr>
<td><strong>Vitamin D2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>35</td>
<td>29.48±22.89</td>
<td>30</td>
<td>45.54±16.51</td>
<td>27</td>
<td>50.30±14.73</td>
</tr>
<tr>
<td>Caucasian</td>
<td>98</td>
<td>61.04±29.43</td>
<td>93</td>
<td>72.02±21.05</td>
<td>91</td>
<td>73.64±20.73</td>
</tr>
<tr>
<td><strong>Vitamin D3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>38</td>
<td>23.90±18.93</td>
<td>24</td>
<td>52.93±14.13</td>
<td>22</td>
<td>60.86±16.99</td>
</tr>
<tr>
<td>Caucasian</td>
<td>99</td>
<td>60.25±25.27</td>
<td>97</td>
<td>85.60±23.02</td>
<td>92</td>
<td>90.64±23.89</td>
</tr>
</tbody>
</table>

SD = standard deviation, Δ change (V1-V3) = total change in 25OHD from V1 to V3, % change (V1-V3) = percentage change in 25OHD from V1 to V3, a Mann-Whitney U Test between South Asian and Caucasian, b Kruskal-Wallis Test, between intervention groups, c Mann-Whitney post-hoc (sig adjusted to p<0.017), d Mann-Whitney post-hoc analysis between all interventions, e Friedman Test (significance at p<0.05) with post-hoc Wilcoxon Signed Rank Test (sig adjusted to p<0.017): 1 significantly different from week 0 in post-hoc analyses, 2 significantly different from week 6 in post hoc analyses.
3.3.8.1 Change in 25OHD Concentrations over Time

These data are shown in Table 3.6 and displayed graphically in Figure 3-3.

3.3.8.1.1 Placebo Intervention

Over the study period both the South Asian and Caucasian placebo cohorts showed a significant decrease in 25OHD concentrations (p=0.023 and p<0.001 respectively). In the South Asian women week 6 25OHD was significantly lower than at baseline (p=0.004), whereas 25OHD at week 12 only showed a trend towards being significantly lower than at baseline (p=0.079) and was not significantly different from week 6. Whereas in the Caucasian women, both week 6 and week 12 25OHD were significantly lower than at baseline (p<0.001 in both cases), and week 12 concentrations were even lower than at week 6 (p<0.001).

3.3.8.1.2 Vitamin D Interventions

As shown in Figure 3-3, in both ethnic groups cohorts, the vitamin D₂ and vitamin D₃ interventions led to a significant increase in 25OHD concentrations (p<0.001). In all cases, 25OHD at week 6 and week 12 were significantly higher than at baseline (p<0.001 in all cases), and it was only within the Caucasian vitamin D₃ intervention group that 25OHD concentrations were significantly higher at week 12 than at week 6 (p<0.001).

3.3.8.1.3 Total and Percentage Change in 25OHD

In both the South Asian and Caucasian groups, total and percentage increase in 25OHD was significantly higher in vitamin D₂ and vitamin D₃ intervention groups than the placebo group (p<0.001 in all cases), as shown in Table 3.6. Both total and percentage change were also higher in the vitamin D₃ intervention than the vitamin D₂ in the Caucasian cohort (p<0.001 in both cases) and a trend towards significance was shown in the South Asian cohort (p=0.036 and p=0.035, respectively).

3.3.8.2 25OHD Concentrations at each Visit

At baseline there were no significant differences in 25OHD concentrations between the three intervention groups, within each ethnic group. However within each intervention group the South Asian cohort had consistently significantly lower 25OHD concentrations than the Caucasians (Table 3.6).
As Table 3.6 also shows, at both week 6 and week 12, the 25OHD concentrations within both the South Asian and Caucasian women were significantly higher in the vitamin D2 intervention group and the vitamin D3 intervention group compared to the placebo group (p<0.001 in all cases). Also at week 6 and week 12, the vitamin D3 intervention group also had significantly higher 25OHD concentrations than the vitamin D2 intervention group in the Caucasian women only (p<0.001 in both cases). The same significant differences were shown for total change and percentage change in 25OHD (Table 3.6).

3.3.8.3 Comparing Ethnic Groups

In the placebo intervention, the Caucasian cohort had a significantly greater decrease in 25OHD (total change in 25OHD) than the South Asian cohort (-15.28 and -5.09 nmol/L, respectively p=0.004). However, the Caucasian women did start with a higher baseline 25OHD concentration (58.8 and 30.8 nmol/L respectively, p<0.001) and at week 12 the Caucasian participants still had a significantly higher 25OHD status than the South Asian group (43.9 and 19.5 nmol/L respectively, p<0.001).

Total change in 25OHD was not significantly different between the South Asian and Caucasian women within the vitamin D2 and vitamin D3 groups, as shown in Table 3.6. However, there was a significant difference in percentage change between the South Asian and Caucasian cohorts in both the vitamin D2 group (112.0% vs 38.6% respectively, p=0.002) and the vitamin D3 group (242.6% vs 71.7% respectively, p<0.001), likely due to the significant difference in 25OHD concentrations at baseline between the South Asian and Caucasian women.

3.3.9 Raising Individuals out of Vitamin D ‘Deficiency’ and ‘Insufficiency’

3.3.9.1 Deficient at Baseline (<25 nmol/l)

As shown in Figure 3-4, in the South Asian women with serum 25OHD <25 nmol/l at baseline, the 12-week vitamin D2 and D3 interventions led to 75% and 100% of participants achieving >25 nmol/L, 33% and 86% of participants achieving >50 nmol/L, and 0% and 14% of participants achieving >75 nmol/l respectively. In the equivalent Caucasian women, both the vitamin D2 and D3 interventions led to 100% of participants achieving >50 nmol/L, and 33% of participants in the D3 intervention achieving >75 nmol/L.
### 3.3.9.2 Insufficient at Baseline (25-50 nmol/L)

As shown in Figure 3-5, in the South Asian women who had a serum 25OHD at baseline between 25-50 nmol/L, both the vitamin D<sub>2</sub> and D<sub>3</sub> 12-week interventions led to 67% of participants achieving serum 25OHD >50 nmol/L, with 33% of participants still remaining within the insufficient range in both cases. In the Caucasian women the vitamin D<sub>2</sub> and D<sub>3</sub> interventions respectively led to 77% and 100% of participants achieving serum 25OHD >50 nmol/L, with 3% and 47% of participants achieving >75 nmol/L. It was only in the vitamin D<sub>2</sub> intervention group that 20% of participants still remained within the insufficient range (25-50 nmol/L). 

Figure 3-5 Percentage of participants within each of the serum 25OHD cut-offs at week 12, who were ‘insufficient’ (25-50 nmol/L) at baseline
3.3.9.3 **Effect of Placebo Intervention**

In the placebo group, at week 12, the percentage of South Asian and Caucasian women with serum 25OHD <25 nmol/l were 86% and 16% respectively, <50 nmol/l were 100% and 58% respectively, and <75 nmol/l were 100% and 96% respectively.

3.3.10 **Change in Metabolites: 25OHD$_2$ and 25OHD$_3$**

These data are shown in Table 3.7 and displayed graphically in Figure 3-6 for 25OHD$_2$ concentrations and Figure 3-7 for 25OHD$_3$ concentrations.

3.3.10.1 **At Baseline: Week 0**

In both the South Asian and Caucasian cohorts there were no significant differences in 25OHD$_2$ concentrations between the three intervention groups (p=0.513 and p=0.592 respectively). This was also the case for 25OHD$_3$ concentrations (p=0.291 and p=0.959 respectively) (Table 3.7).

Within intervention groups, both 25OHD$_2$ and 25OHD$_3$ concentrations were significantly lower in the South Asian women than the Caucasian women (p<0.005), except in the placebo intervention groups where the South Asian women had significantly higher 25OHD$_2$ concentrations (p=0.017).

3.3.10.2 **At Week 6 and 12**

At visits 2 and 3, within both the South Asian and Caucasian cohorts, 25OHD$_2$ concentrations were significantly higher in the vitamin D$_2$ intervention group than both the placebo (p<0.002 in all cases) and vitamin D$_3$ intervention groups (p<0.001 in all cases), as was the total change and percentage change in 25OHD$_2$ (p<0.001 in all cases). In the Caucasian cohort only, 25OHD$_2$, total change in 25OHD$_2$ and percentage change in 25OHD$_2$ was significantly lower in those on the vitamin D$_3$ intervention than those on the placebo intervention, at both week 6 and week 12 (p<0.001 in all cases).

At visits 2 and 3 within both the South Asian and Caucasian cohorts, 25OHD$_3$ concentrations were significantly higher in the vitamin D$_3$ intervention group than both the placebo (p<0.001 in all cases) and vitamin D$_2$ intervention groups (p<0.001 in all cases), as was the total change and percentage change in 25OHD$_3$ (p<0.001 in all cases). In the Caucasian cohort only, 25OHD$_3$, total change in 25OHD$_3$ and percentage change in 25OHD$_3$ was significantly lower in those on the vitamin D$_2$
intervention than those on the placebo intervention, at both week 6 and week 12 (p<0.001 in all cases).

3.3.10.3 Changes Over Time

As shown in Figure 3-6 and Figure 3-7, in the placebo intervention groups 25OHD$_2$ concentrations did not change over time in either ethnic cohort, whereas 25OHD$_3$ concentrations in both the South Asian and Caucasian cohorts were significantly lower at week 6 (p=0.004 and p<0.001 respectively). At week 12 the South Asian cohort showed a trend towards having a significantly lower 25OHD$_3$ concentration than at baseline, whereas the Caucasian cohort continued to show a significant difference from baseline (p=0.055 and p<0.001 respectively) (Table 3.7).

In the vitamin D$_2$ intervention group the 25OHD$_2$ concentrations were significantly higher at both week 6 and week 12 than at baseline, in both the South Asian and Caucasian group (p<0.001 in all cases), whereas the 25OHD$_3$ concentrations were significantly lower (p<0.001 in all cases), as shown in Figure 3-6 and Figure 3-7 (Table 3.7).

In the vitamin D$_3$ intervention group the 25OHD$_3$ concentrations were significantly higher at both week 6 and week 12 compared to baseline, in both the South Asian and Caucasian cohorts (p<0.001 in all cases). The Caucasian cohort showed significantly lower 25OHD$_2$ concentrations at visits 2 and 3 compared to baseline (p<0.001 in both cases), as shown in Figure 3-6 and Figure 3-7 (Table 3.7).
Figure 3-6 Serum 25OHD2 concentrations of the South Asian and Caucasian women at all three visits, split by interventions (juice and biscuit groups combined)
Significant differences between groups, at each time point, are shown in Table 3.7

Figure 3-7 Serum 25OHD3 concentrations of the South Asian and Caucasian women at all three visits, split by interventions (juice and biscuit groups combined)
Significant differences between groups, at each time point, are shown in Table 3.7
Table 3.7 Serum 25OHD$_2$ and 25OHD$_3$ concentrations across the three study visits in the placebo, vitamin D$_2$ and vitamin D$_3$ intervention groups, split by ethnicity

<table>
<thead>
<tr>
<th></th>
<th>Week 0 (V1)</th>
<th>Week 6 (V2)</th>
<th>Week 12 (V3)</th>
<th>Δ change (V1-V3)</th>
<th>% change (V1-V3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S Asian</td>
<td>17</td>
<td>3.35 ±8.61</td>
<td>27.42 ±27.37</td>
<td>15</td>
<td>3.07 ±7.82</td>
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<td>Cauc</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>p-value$^a$</td>
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<td>0.002</td>
<td>&lt;0.001</td>
<td>0.004</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cauc</td>
<td>98</td>
<td>2.38 ±2.47</td>
<td>58.66 ±29.37</td>
<td>93</td>
<td>36.30 ±12.60</td>
</tr>
<tr>
<td>p-value$^a$</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Vitamin D$_3$</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S Asian</td>
<td>38</td>
<td>1.26 ±1.11</td>
<td>22.65 ±18.93</td>
<td>24</td>
<td>1.17 ±1.16</td>
</tr>
<tr>
<td>Cauc</td>
<td>99</td>
<td>2.13 ±1.91</td>
<td>58.12 ±24.96</td>
<td>97</td>
<td>1.74 ±1.54</td>
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<tr>
<td>p-value$^a$</td>
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<td></td>
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<tr>
<td>S Asian</td>
<td>0.513</td>
<td>0.291</td>
<td>&lt;0.001$^2$</td>
<td>&lt;0.001$^3$</td>
<td>&lt;0.001$^2$</td>
</tr>
<tr>
<td>Cauc</td>
<td>0.592</td>
<td>0.959</td>
<td>&lt;0.001$^2$</td>
<td>&lt;0.001$^* $</td>
<td>&lt;0.001$^2$</td>
</tr>
</tbody>
</table>

SD = standard deviation, Δ change (V1-V3) = total change in 25OHD from V1 to V3, % change (V1-V3) = percentage change in 25OHD from V1 to V3, $^a$ Mann-Whitney U Test between South Asian and Caucasian, $^b$ Kruskal-Wallis Test, between intervention groups, $^c$ Mann-Whitney post-hoc (sig adjusted to p<0.017): $^* p<0.001$ in post-hoc analyses between vit D2 and both placebo and vit D3 intervention, $^2 p<0.001$ in post-hoc analyses between vit D3 and both placebo and vit D2 intervention, $^3 p<0.001$ in post-hoc analysis between all interventions.
3.3.11 Week 16 Results: 4 Weeks Post-Intervention

A fourth study visit was completed in a total of 38 Caucasian participants. These subjects were selected based on having 100% compliance and having completed the 12-week intervention within a sufficient time frame that meant the fourth visit would be completed before the end of March.

3.3.11.1 Total 25OHD

The serum total 25OHD results from this sub-group of participants, across all four visits, are shown in Table 3.8 and Figure 3-8. At week 16 the 25OHD concentrations in the vitamin D\(_2\) and vitamin D\(_3\) intervention groups were significantly lower than at week 12 (p=0.002 and p<0.001, respectively). The 25OHD concentrations at week 16 were also significantly lower than at baseline in the placebo intervention group (p=0.008) and significantly higher than at baseline in the vitamin D\(_3\) intervention group (p=0.003). However, in the vitamin D\(_2\) intervention group the 25OHD concentrations at week 16 were not significantly different from baseline.

Table 3.8 Serum 25OHD concentrations, and change in 25OHD, across the four study visits in the sub-set of Caucasian participants (n=38)

<table>
<thead>
<tr>
<th></th>
<th>Total 25OHD Concentration (nmol/L)</th>
<th>Δ Change in 25OHD</th>
<th>% Change in 25OHD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 0 (V1)</td>
<td>Week 6 (V2)</td>
<td>Week 12 (V3)</td>
</tr>
<tr>
<td>Placebo</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>9</td>
<td>57.6 ±9.92</td>
<td>49.96 ±11.65</td>
<td>41.3 ±12.74</td>
</tr>
<tr>
<td>Vitamin D(_2)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>14</td>
<td>55.13 ±21.29</td>
<td>66.25 ±17.18</td>
<td>71.34 ±19.00</td>
</tr>
<tr>
<td>Vitamin D(_3)</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>15</td>
<td>66.15 ±22.17</td>
<td>86.45 ±20.45</td>
<td>93.17 ±22.45</td>
</tr>
<tr>
<td>Between Treatment Groups</td>
<td>K-W p-value</td>
<td>0.217</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

K-W= Kruskal-Wallis test, followed by Mann-Whitney U-Test post-hoc: a sig diff from placebo intervention (p<0.017), b sig diff from vitamin D\(_2\) intervention (p<0.017), c sig diff from vitamin D\(_3\) intervention (p<0.017); Friedman tests followed by post-hoc Wilcoxon Signed Ranks tests: 1 sig diff from week 0 (p<0.008), 2 sig diff from week 12 (p<0.002)

3.3.11.2 25OHD\(_2\) and 25OHD\(_3\) Concentrations at Week 16

As shown in Figure 3-9, 25OHD\(_2\) concentrations remained unchanged between week 12 and week 16 in the placebo treated group, whereas within the vitamin D\(_2\) treated group 25OHD\(_2\) concentrations decrease from 45.06 ±17.5 nmol/L at week 12 to 22.40 ±8.8 nmol/L at week 16 (p<0.001), and in the
vitamin D₃ intervention group 25OHD₂ concentrations increased from 1.57 ±1.0 nmol/L at week 12 to 2.55 ±1.6 nmol/L at week 16 (p<0.001). 25OHD₂ concentrations at week 16 were only significantly different from baseline within the vitamin D₂ intervention group (1.74 ±1.0 to 22.40 ±8.8 nmol/L, p<0.001).

As shown in Figure 3-10, 25OHD₃ concentrations decreased between week 12 and week 16 in the vitamin D₃ intervention group (91.6 ±21.9 to 74.6 ±19.6 nmol/L, p<0.001), but increased between week 12 and week 16 within the vitamin D₂ intervention group (26.3 ±20.1 to 33.6 ±22.4 nmol/L, p<0.001). At week 16, 25OHD₃ concentrations remained significantly higher than at baseline in the vitamin D₃ intervention group (74.6 ±19.6 vs 63.6 ±22.0 nmol/L, p=0.003), and significantly lower than at baseline in the placebo (37.9 ±13.9 vs 55.34 ±10.7 nmol/L, p=0.008) and vitamin D₂ interventions (53.4 ±21.6 vs 33.6 ±22.4 nmol/L, p=0.005).

Figure 3-8 Serum total 25OHD concentrations over the four study visits (weeks 0-16) in sub-set of Caucasian participants
*indicates significant difference between week 0 and week 12 (p<0.008), **indicates significant difference between week 12 and week 16 (p<0.002)
Figure 3-9 Serum total 25OHD$_2$ concentrations over the four study visits (weeks 0-16) in sub-set of Caucasian participants
*indicates significant difference between week 0 and week 12 (p<0.001), **indicates significant difference between week 12 and week 16 (p<0.001)

Figure 3-10 Serum total 25OHD$_3$ concentrations over the four study visits (weeks 0-16) in sub-set of Caucasian participants
*indicates significant difference between week 0 and week 12 (p<0.008), **indicates significant difference between week 12 and week 16 (p<0.001)
3.3.12 Vitamin D Binding Protein

In a sub-set of 18 South Asian and 54 Caucasian participants, vitamin D binding protein (VDBP) was measured. These participants were selected based on their concomitant inclusion in the gene expression analyses, as described in Chapter 6.2.1.

3.3.12.1 Baseline Vitamin DBP

At baseline there was no significant difference between the South Asian and Caucasian VDBP concentrations (362.13 ±142.85 vs 384.81 ±158.98 respectively, p=0.917), nor were there significant differences between intervention groups within each ethnic group (Table 3.9).

### Table 3.9 Vitamin D binding protein (VDBP) concentrations in both South Asian and Caucasian women in the placebo, vitamin D$_2$ and vitamin D$_3$ intervention groups, across the three study visits

<table>
<thead>
<tr>
<th></th>
<th>Week 0 VDBP (μg/ml)</th>
<th>Week 6 VDBP (μg/ml)</th>
<th>Week 12 VDBP (μg/ml)</th>
<th>Friedman p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>6</td>
<td>404.0 ±155.3</td>
<td>6</td>
<td>454.9 ±88.3</td>
</tr>
<tr>
<td>Caucasian</td>
<td>18</td>
<td>363.2 ±154.8</td>
<td>18</td>
<td>366.6 ±130.8</td>
</tr>
<tr>
<td><strong>Vitamin D2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>6</td>
<td>275.0 ±133.4</td>
<td>6</td>
<td>293.2 ±171.4</td>
</tr>
<tr>
<td>Caucasian</td>
<td>18</td>
<td>351.9 ±124.6</td>
<td>18</td>
<td>353.8 ±146.4</td>
</tr>
<tr>
<td><strong>Vitamin D3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>6</td>
<td>407.4 ±117.6</td>
<td>6</td>
<td>420.0 ±59.3</td>
</tr>
<tr>
<td>Caucasian</td>
<td>18</td>
<td>439.3 ±185.9</td>
<td>18</td>
<td>425.3 ±182.3</td>
</tr>
<tr>
<td><strong>K-W Test p-value</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>0.173</td>
<td>0.181</td>
<td>0.064</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.353</td>
<td>0.481</td>
<td>0.620</td>
<td></td>
</tr>
</tbody>
</table>

Key: SD=standard deviation, K-W=Kruskal-Wallis test, significance at p<0.05

3.3.12.2 Change in VDBP Concentrations

Within each ethnic group there was no significant difference in VDBP concentrations between intervention groups at any time point (week 0, 6 and 12), nor was there any significant change in VDBP concentrations over time within each intervention group split by ethnicity (Table 3.9).

There was no significant difference in total change in VDBP between any of the intervention groups (p=0.720 , see Figure 3-11 for means).
3.4 DISCUSSION

3.4.1 Vitamin D$_2$ vs. Vitamin D$_3$

The main aim of this study was to compare the efficiency of 15 µg/d (600 IU/d) of vitamin D$_2$ vs. vitamin D$_3$ fortification of food products in raising serum 25OHD concentrations in South Asian and Caucasian women. This study shows that daily supplementation with vitamin D$_3$ led to a significantly higher total 25OHD concentration by week 12 than the vitamin D$_2$ intervention, in both the South Asian and Caucasian adult population, with vitamin D$_2$ producing a 18 nmol/L and 12 nmol/L increase in total 25OHD, and vitamin D$_3$ leading to a 33 nmol/L and 30 nmol/L increase in total 25OHD, in the South Asian and Caucasian women, respectively.

This is the largest single study to compare the efficacy of vitamin D$_2$ and vitamin D$_3$ in raising serum 25OHD concentrations. A total of 118 participants completed the vitamin D$_2$ intervention (27 South Asian and 91 Caucasian) and 114 participants completed the vitamin D$_3$ intervention (22 South Asian and 92 Caucasian). In previous studies, the number of participants within each intervention arm ranged from 10-55, although in most cases it was <20 per intervention. Although these study numbers are not as high as those in the meta-analysis (n150 in the vitamin D$_2$ and n194 in the...
vitamin D3; Tripkovic et al. 2012), the 118 and 114 participants within the D2-D3 Study all followed the same protocol, whereas the meta-analysis was a group of very heterogeneous small studies.

As previous studies comparing vitamin D$_2$ to D$_3$ have variable study designs, comparison of the current study with those of previous studies needs to be undertaken with caution. Comparing this study to the main findings of a meta-analysis, published in 2012, that compared the efficacy of vitamin D$_2$ vs D$_3$ on 25OHD concentrations (Tripkovic et al. 2012), both the current D2-D3 study and the meta-analysis found that vitamin D$_3$ was more effective than vitamin D$_2$. However, in the meta-analysis, this finding was mainly driven by the studies giving large bolus doses of vitamin D (Romagnoli et al. 2008; Binkley et al. 2011; Heaney et al. 2011). When the studies giving smaller daily doses of vitamin D (25-100μg/d (1,000-4,000IU/d)) were analysed individually, there was no significant difference between the D$_2$ and D$_3$ intervention in raising 25OHD concentrations (Trang et al. 1998; Holick et al. 2008; Glendenning et al. 2009; Biancuzzo et al. 2010; Binkley et al. 2011). A reason why these daily dosing studies when combined did not show a significant difference between the two forms of vitamin D, despite giving a higher dose of vitamin D than the current study, is through a lack of study power; all six studies included had <20 participants per group (except for 1 group of n55 within one arm of the intervention).

Of the 10 previous intervention studies comparing vitamin D$_2$ and vitamin D$_3$ on 25OHD concentrations that have given daily dosing, seven of them found that D$_3$ was more effective than D$_2$ (Tjellesen et al. 1986; Trang et al. 1998; Glendenning et al. 2009; Binkley et al. 2011; Logan et al. 2013; Lehmann et al. 2013; Oliveri et al. 2015) but three found no difference in the efficacy of the two forms (Holick et al. 2008; Biancuzzo et al. 2010; Fisk et al. 2012). However, these studies have serious flaws in the study design, with study number below 20 within each intervention group, and the authors omitted data in the paper, which would be vital for the accurate interpretation of results (Lanham-New et al. 2010).

Since the meta-analysis, there have been four more studies comparing the efficacy of D$_2$ and D$_3$ on raising 25OHD concentrations (Fisk et al. 2012; Logan et al. 2013; Lehmann et al. 2013; Oliveri et al. 2015). Logan and colleagues (2013) demonstrated that 25 μg/d vitamin D$_3$ was able to maintain 25OHD concentrations, when the placebo and vitamin D$_2$ interventions led to a significant decrease in 25OHD, even when baseline 25OHD concentrations are significantly higher (80 nmol/L) than in the current D2-D3 Study. Lehmann and colleagues (2013) also demonstrated that vitamin D$_3$ was more effective than vitamin D$_2$ at raising total 25OHD, when given as a daily dose of 50 μg/d. These data
are consistent with the D2-D3 Study findings, as the magnitude of change in 25OHD was higher which would be expected given the dose of vitamin D given was higher.

Fisk and colleagues (2012) compared fortified malted milk drinks with vitamin D$_2$ and D$_3$ in doses of 5 and 10 μg/d for 4 weeks in a total of 40 men and women; 10 per intervention. They concluded that vitamin D$_2$ and vitamin D$_3$ are equipotent at raising total 25OHD concentrations, although they fail to compare total 25OHD concentrations. They do show that the rise in 25OHD$_2$ and 25OHD$_3$ in response to vitamin D$_2$ and vitamin D$_3$, respectively, did not differ. However, they did not compare total 25OHD or change in 25OHD between the vitamin D$_2$ and vitamin D$_3$ interventions, even though the means (95%CI) suggest there may be differences; 5 μg/d vitamin D$_2$ = 4.9 (-2.3,12.7) nmol/L, 5 μg/d vitamin D$_3$ = 11.9 (2.7,21.2) and 10 μg/d vitamin D$_2$ = 13.6 (4.1,23.0) nmol/L, 10 μg/d vitamin D$_3$ = 19.7 (9.4,30.1) nmol/L. This highlights the importance of measuring total 25OHD, 25OHD$_2$ and 25OHD$_3$ when determining the comparative efficacy of vitamin D$_2$ and D$_3$, in building a clear picture and exploring the mechanisms.

### 3.4.1.1 Changes in 25OHD$_2$, 25OHD$_3$ and VDBP – Mechanisms for Differences

In the current study, we saw an increase in 25OHD$_2$ in the vitamin D$_2$ treated group, and an increase in 25OHD$_3$ in the vitamin D$_3$ treated group, as was expected and is consistent with previous studies (Armas et al. 2004; Glendenning et al. 2009; Biancuzzo et al. 2011; Fisk et al. 2012; Lehmann et al. 2012; Logan et al. 2013). Within the vitamin D$_2$ treated groups 25OHD$_3$ concentrations decrease, which has also been shown previously (Tjellson et al. 1986; Armas et al. 2004; Binkley et al. 2011; Lehmann et al. 2012), although not consistently (Glendenning et al. 2009; Biancuzzo et al. 2011; Fisk et al. 2012). In cross-sectional analyses, higher 25OHD$_2$ concentrations have also been associated with lower 25OHD$_3$ concentrations (Swanson et al. 2014). The decline in 25OHD$_3$ in those taking vitamin D$_2$ is a potential mechanism for explaining how vitamin D$_3$ is more effective than vitamin D$_2$ at raising 25OHD concentrations. The decrease in 25OHD$_3$ could reflect either competition by vitamin D$_2$ for the binding to 25-hydroxylase (Holmerg et al. 1986) or the vitamin D binding protein (VDBP) (Houghton & Veith 2006) resulting in a decreased metabolism of vitamin D$_3$, or increased degradation of the 25OHD$_3$ by a mechanism that is up regulated to metabolise vitamin D$_2$, or both.

It was hypothesized that VDBP concentrations would increase in the D$_2$ and D$_3$ intervention groups, with an increase in 25OHD concentrations due to increase demands on the VDBP for transporting the vitamin D and the metabolites, nevertheless, VDBP concentrations did not change over time. It is possible that there may be changes in the amount of free and bioavailable 25OHD concentrations, which can be calculated from VDBP concentrations in future work.
3.4.2 Vehicle for Fortification

An aim of this study was to determine which vehicle for fortification (orange juice as ‘liquid’ vs. biscuit as ‘solid’) with vitamin D$_2$ vs. vitamin D$_3$ is more effective in raising 25OHD concentrations, independent of ethnicity. Participants were asked to consume the intervention products on their own, and not with a meal, so that we could discriminate between the effects of the different nutritional composition of the juice and the biscuit on the response to the vitamin D$_2$ and D$_3$. It was hypothesised that the biscuit would be a more suitable food vehicle than the orange juice, resulting in a greater increase in 25OHD, due to the fat content within the biscuit, and not within the orange juice, aiding absorption of vitamin D (Basu et al. 2003). Previous studies have shown that a bolus dose of 1,250 μg (50,000 IU) vitamin D$_3$ consumed with a fat-containing meal (30% calories from fat) leads to a 32% greater peak in plasma vitamin D$_3$ after 12-hours than when consumed with a fat-free meal (Dawson-Hughes et al. 2015). However, the D2-D3 Study showed no difference between an orange juice and a biscuit as the food vehicle for vitamin D$_2$ or vitamin D$_3$, in raising 25OHD concentrations. Similar findings have been shown following a bolus dose of 1,250 μg vitamin D$_3$, where no significant difference between 25OHD concentrations at 30 days and 90 days post consumption of vitamin D$_3$ with a no-fat, low-fat or high-fat meal has been shown (Dawson-Hughes et al. 2013). This would suggest that the fat content of a food used for fortification, or the dietary fat consumed at the time of the vitamin D, does not impact on the bioavailability of either vitamin D$_2$ or vitamin D$_3$. A study has however shown that dietary fat consumption, specifically mono-unsaturated fatty acids (MUFA), over the period of supplementation (and not at the time of supplement consumption) is associated with change in 25OHD, with MUFA intakes being positively associated with the change in 25OHD concentrations (Niramitmahapanya et al. 2011).

To elucidate whether daily dietary fat intake is relevant, studies comparing vitamin D supplementation/fortified foods consumption on high, normal and low fat diets would be needed. However, this dietary approach would be confounded by the effects of weight change.

In this study, the orange juice and biscuit were not compared to supplementation as the study focused on food fortification as a population-wide solution for improving 25OHD concentrations. However, orange juice as a vehicle for vitamin D supplementation has previously been used and was shown to be equally effective as a supplemental form of vitamin D (Biancuzzo et al. 2010).
3.4.3  Response to Dose

A further aim of this study was to investigate if 15 µg/d was effective in raising wintertime 25OHD above ‘deficiency/insufficiency’ thresholds (25 nmol/l and 50 nmol/l respectively) and if there are differences in vitamin D₂ vs. vitamin D₃ fortification. This study showed that vitamin D₃ is more effective than vitamin D₂ at raising concentrations above the thresholds of ‘deficiency’ and ‘insufficiency’. The D₃ intervention was able to raise 100% of the South Asian and Caucasian women above the deficiency threshold, and even 100% of the Caucasian women above the insufficiency threshold, whereas the D₂ intervention was able to raise 100% of the Caucasian women above the deficiency threshold, but 11% of the South Asian women remained below the deficient threshold.

3.4.4  Vitamin D Concentrations at Week 16

At week 16, the vitamin D₂ intervention 25OHD concentrations had already returned to baseline concentrations, whereas 25OHD concentrations remained significantly higher than at baseline in the vitamin D₃ intervention. Vitamin D₃ is therefore not only able to increase total 25OHD more than vitamin D₂, but can also maintain 25OHD concentrations for a more prolonged period, suggesting that the degradation of 25OHD₂ is higher than 25OHD₃ when there is no vitamin D supplementation or fortification. This could be due to 25OHD₂ having a shorter half-life than 25OHD₃ (Jones et al. 2014).

In the vitamin D₂ and vitamin D₃ interventions, 25OHD₂ and 25OHD₃ concentrations decreased, respectively between week 12 and week 16. However, in the vitamin D₂ intervention group 25OHD₃ concentrations began to increase again post-intervention. This provides further evidence to suggest that vitamin D₂ may compete with vitamin D₃ for the binding to 25-hydroxylase (Holmerg et al. 1986) or the VDBP (Houghton & Veith 2006), and/or cause increased degradation of the 25OHD₃ by a mechanism that is up-regulated to metabolise vitamin D₂. When the vitamin D₂ intervention was complete, the metabolism of vitamin D₃ to 25OHD₃ increased again, and/or the degradation of 25OHD₃ was no longer up-regulated.

3.4.5  Strengths of Study

A strength of this study was the number of participants, given that it is the largest randomised, controlled trial comparing the efficacy of vitamin D₂ vs. vitamin D₃ on raising serum 25OHD concentrations, and its inclusion of the South Asian population.
The study design was also a strength; the study was double-blinded so that neither researcher nor participant knew which intervention each participant was on, and the study period was restricted to the months of October to March, thereby eliminating the influence of sun exposure on serum 25OHD.

The serum 25OHD concentrations were measured using the gold-standard measurement technique of LC-MS/MS allowing for the accurate and precise quantification of 25OHD₂ and 25OHD₃. The laboratory used is accredited by the Clinical Pathology Accreditation (CPA) UK (CPA number 0865) and has been certified as proficient by the Vitamin D Quality Assurance Scheme (DEQAS). The measurement of 25OHD₂ and 25OHD₃ concentrations allowed for a greater understanding of the potential mechanism by which the two different forms of vitamin D may be absorbed or metabolised differently in the human body.

3.4.6 Limitations

The diet diaries were inadequate tools for collecting dietary intakes within the South Asian women, as there was only a 37% response rate. Drop-out rates amongst the South Asian women were also higher at 30%. English was the second language for most of the South Asian women who were lost to follow up, so perhaps having Urdu/Arabic interpreters at appointments would have been beneficial for rapport building and communication of information, such as what the diet diaries were for and how to complete them, which may have improved response and retention rates.

Due to time limitations, and the importance of maintaining sufficient statistical power, this study was limited to the examination of two ethnic groups; South Asian and Caucasian. For the same reason, the study was also limited to only females. Similar studies within different population groups such as children, elderly, males, ethnic groups and obese populations may have produced different results.

If time and cost were not a limiting factor, it would have been useful to have had two different doses of vitamin D₂ and vitamin D₃, so that a dose-response element could have been brought into the analyses.
3.4.7 Further Research

Follow-up work on this current project will be to calculate free and bioavailable serum 25OHD concentrations, using the VDBP concentrations, to look at whether they change in response to vitamin D$_2$ or vitamin D$_3$, and the association with 25OHD concentrations. There is also scope for more detailed analysis of dietary intakes, and whether dietary intakes or changes in diet over the study period were associated with response to vitamin D$_2$ and/or vitamin D$_3$.

As this study was undertaken in South Asian and Caucasian female adults, it would be interesting to see whether the findings can be reproduced in different age, gender and ethnic groups. It would also be useful to look at response to different doses of vitamin D in order to conduct a dose-response analysis. In particular, it would be interesting to see the response to 10 µg/d which is the proposed daily recommendation in the UK (SACN 2015).

Within the field of food fortification as a scheme for population-wide improvement of vitamin D status, further research into the efficacy and safety of fortifying staple foods such as bread and yoghurt would be useful. Examining the fortification of foods commonly consumed within ethnic groups at risk of vitamin D deficiency, such as South Asian women, as a method of targeting those at risk would also be an interesting project. For example, as a method of targeting the South Asian cohort, chapatti flour could be fortified with vitamin D. There are also an increasing number of products available that have not been fortified with vitamin D, but have been enriched i.e. yeast exposed to UVB in order to increase vitamin D$_3$ that can then be delivered via bread. Comparing these enrichment methods with fortification would also be useful, particularly for government organisation and policy makers, but also for the general public who will be consuming such products.

3.4.8 Conclusion

In conclusion, the present study indicates that both vitamin D$_2$ and vitamin D$_3$, delivered through fortified foods at a dose of 15 µg/d (600IU/d), raise total serum 25OHD concentrations over 12-weeks in the winter-time. However, vitamin D$_3$ was shown to produce a greater increase in total serum 25OHD and had a greater capacity to achieve ‘sufficient’ (>50 nmol/l) 25OHD concentrations across both UK dwelling South Asian and Caucasian female populations.
Chapter 4 - THE D2-D3 BONE STUDY
4.1 INTRODUCTION

4.1.1 Bone Throughout the Life Course

Bone mass changes throughout the lifecycle, as shown below in Figure 4-1. Bone is continuously being remodelled throughout life, principally through the actions of osteoblasts and osteoclasts. Osteoblasts are responsible for bone formation, whereas osteoclasts are involved in bone resorption. Bone mass in later life is predominantly determined by three factors; (1) peak bone mass (PBM) attained during adolescence and early adulthood, (2) bone mass maintenance during adulthood, and (3) the progressive rate of bone loss with age, where 1-2% of bone can be lost over a 5-10 year period, with the menopause being of considerable concern for women as, for some women, over one fifth of bone density will be lost.

![Figure 4-1 Changes in bone mass during the lifecycle.](image)

Peak bone mass (PBM) attainment takes place from 0-28 years, with a critical period of PBM attainment during puberty, followed by age-related bone loss from the age of 40 years, with the menopausal period (menopause and ≤10 years post-menopause) seeing a loss of 1-2% of bone. Taken from Lanham-New (2008).

Optimum bone health throughout the life course is essential for the avoidance of osteoporosis in older age. Osteoporosis is defined by the World Health Organisation as a ‘systemic skeletal disease characterised by low bone mass and micro-architectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture’ (Peck et al. 1993). It is estimated that one in three women and one in twelve men aged >50 years will suffer from osteoporosis in their lifetime (van Staa et al. 2001); making this a condition of high public health importance. A number of factors are known to be associated with bone mass and risk of osteoporosis including age (Raisz and Seeman 2011; Khosla and Riggs 2005), gender (Looker et al. 1995; Berntsen et al. 2001; Dennison et
al. 1999), ethnicity (Finkelstein et al. 2002), genetics (Ralston & Uitterlinden 2010; Kemp et al. 2014; Warrington et al. 2015), obesity (Reid et al. 1992), menopausal status (Finkelstein et al. 2008), alcohol (Schapira, 1990), smoking (Law and Hackshaw, 1997), physical activity (Fuchs et al. 2001; Nurmi-Lawton et al. 2004) and nutritional factors (Lanham-New et al. 2008).

4.1.2 The Role of Vitamin D in Bone Health

The most important role of vitamin D, through the active hormone 1,25(OH)₂D, is to maintain bone calcium, by regulating calcium and phosphorus metabolism, and calcium absorption from the intestine (Lips 2006), as discussed in detail in Chapter 1.3.2.

4.1.3 Assessing Bone Health

Although the most clinically relevant endpoint in assessing bone health would be bone fracture, most studies use intermediate outcome measures to assess bone health and subsequent risk of fracture. These intermediate outcomes include bone strength, biochemical markers and musculoskeletal outcomes.

4.1.3.1 Bone Strength

The most common measurement of bone strength is areal bone mineral density (aBMD) and the most common technique used to measure aBMD is dual x-ray absorptiometry (DXA). However, measuring aBMD has limitations in its assessment of bone strength as it does not take into account other factors that contribute to bone strength such as bone size, shape, architecture and turnover (Ammann & Rizzoli, 2003). Use of aBMD in cross sectional studies is therefore limited, unless adjustments were made for the confounding influence of bone size. Volumetric bone mineral density (vBMD) is a better estimate of bone density, as the measurement is not confounded by bone size. Quantitative computed tomography (QCT) and peripheral quantitative computed tomography (pQCT) completes a three-dimensional assessment of the structural and geometric properties of the bone to assess vBMD. Measuring the effect of vitamin D supplementation on bone parameters such as bone density is difficult in most intervention trials, due to the time needed for the intervention to have any possibility of change. Therefore, bone biochemistry or musculoskeletal functional outcomes can also be used to assess the effect of vitamin D supplementation on bone health.

4.1.3.2 Biochemical Markers

In addition to measuring 25OHD, 1,25(OH)₂D and parathyroid hormone (PTH) concentrations in the assessment of bone health, due to their role in regulating calcium and phosphorus homeostasis, serum total calcium and albumin (to calculate albumin adjusted/corrected calcium) are often
measured to detect conditions associated with hypercalcemia, such as primary hyperparathyroidism or hypocalcemia and consequent secondary hyperparathyroidism which may cause bone loss.

Bone turnover markers in serum can be used to assess bone turnover rate, and therefore the metabolic activity of the skeleton, to predict fracture risk. Both bone formation markers, such as procollagen type 1 N-terminal propeptide (P1NP), and bone resorption markers, such as C-terminal telopeptide of collagen type 1 (CTX), can be measured (Johansson et al. 2014). However, the use of these bone turnover markers is limited as they cannot be used to determine the metabolic activity of different skeletal compartments (Garnero 2014), and they are expensive measurements.

**4.1.3.3 Musculoskeletal Outcomes**

Muscle strength and function has been shown to be an independent predictor of bone mineral density, with decreased muscle strength being associated with lower bone mineral density (Snow-Harter et al. 1990), and is therefore a musculoskeletal outcome that can be measured to assess bone strength and subsequent risk of fracture. Frailty (Ensrud et al. 2007), incidence of falls (Clark et al. 2012) and prior fractures (Gehlbach et al. 2012) are also determinants of fracture risk.

**4.1.4 Vitamin D and Musculoskeletal Health Outcomes**

Vitamin D deficiency has been associated with skeletal conditions and other musculoskeletal health outcomes. In children, rickets, (which is a condition that effects bone development in childhood causing the bones to become soft and weak leading to bone deformities), is associated with low 25OHD concentrations of <12 nmol/L in the majority of cases (SACN 2015) and in adults, osteomalacia, which is the softening of bone in adults, is also shown to only be present when 25OHD concentrations are ≤20 nmol/L (SACN 2015). Epidemiological studies, including randomised-controlled trials, have reported associations between 25OHD concentrations and muscle strength and function (Ward et al. 2010; Tomlinson et al. 2014), fracture risk (Chapuy et al. 1992; Dawson-Hughes, 2008), risk of falls (Mowe et al. 1999; Stein et al. 1999), and stress fracture risk in military personnel (Dao et al. 2015).

Supplementation with vitamin D has been shown to have a positive effect on bone mineral density (BMD) (Dawson-Hughes et al. 1997; Chapuy et al. 1992; Ooms et al. 1995). In this respect, a recent meta-analysis of 23 studies showed a small benefit of vitamin D supplementation on femoral neck BMD (Reid et al. 2014). However, it is important to note that there was heterogeneity among the trials ($I^2=67\%, p<0.00027$).
As shown in Chapter 3.3.8, vitamin D₂ and D₃ are not equally effective at raising 25OHD concentrations and it is reasonable to speculate that they may not have equal effects on bone health. Previous studies, that have shown differential responses in 25OHD concentrations to vitamin D₂ and D₃, have shown no difference in PTH concentrations between the groups (Logan et al. 2013), nor any difference in 1,25(OH)₂D concentrations (Trang et al. 1998). However, very few of these studies have measured or published all three key biochemical markers (25OHD, PTH and 1,25(OH)₂D) together which therefore limits the ability to determine: i) the impact of and ii) differences in impact of vitamin D₂ and D₃ on markers of bone health beyond the effect on 25OHD concentrations.

4.1.5 Ethnic Differences in 25OHD Concentrations and Bone Parameters

In the UK, the South Asian population are one of the groups at risk of vitamin D deficiency as they exhibit risk factors such as having darker skin pigmentation and cultural customs, which limit skin exposure. The higher risk of vitamin D deficiency year round (Darling et al. 2013a) suggests these populations may also be at a higher risk of poor bone health, although studies of vitamin D in relation to bone density within such ethnic groups are limited (Cranney et al. 2007). In the few studies conducted in UK dwelling South Asian populations, bone mineral density has been shown to positively correlate with 25OHD concentrations and negatively correlate with PTH concentrations at the hip and spine (Roy et al. 2007). Vitamin D deficiency (<25 nmol/L) has also been associated with widespread pain (Macfarlane et al. 2005).

There is some evidence of differences in fracture risk between ethnic groups, and South Asian women have been shown to have a higher incidence of osteoporosis at the femoral neck, when compared to Caucasian women (Khandewal et al. 2012). Bone structure and microarchitecture varies between many ethnic groups, as discussed in a review by Zengin et al. (2015). Within the context of this work, only the South Asian and Caucasian ethnic groups are discussed. Traditionally, areal bone mineral density (aBMD) has been used to assess bone strength and fracture risk, and studies that have used aBMD to assess bone strength differences between ethnic groups do not consistently parallel fracture rates, as it does not take into account the bone size which is thought to differ between South Asian and Caucasian cohorts (Darling et al. 2013b). While studies assessing bone structure by QCT in UK dwelling South Asian cohorts are limited, in pre-menopausal women there was significantly lower vBMD and BMC in the South Asian compared to Caucasian cohort, though bone strength as assessed by strength strain index (SSI) was not different between ethnic groups. This suggests a possible adaption to lower vBMD and BMC within the South Asian population (Ward et al. 2007). However, in contrast to this Darling and colleagues (2013b) found that South Asian
women had significantly smaller bones, but higher vBMD at both the 4% radius and 4% tibia compared to Caucasian cohorts, and they also showed poorer bone strength when assessed by SSI and fracture load, suggesting a detrimental effect of smaller bones on bone strength and fracture risk.

Few studies have assessed the relationship between vitamin D status and bone geometry using pQCT methods, and even fewer have done so in South Asian cohorts. In the few studies that have been done in Caucasians, a relationship between vitamin D status and radial mass (Darling et al. 2012), distal radial total area (Darling et al. 2012), radial mid-shaft cortical area (Darling et al. 2012), radial cortical vBMD (Cheng et al. 2003), radial total bone density (Boonen et al. 1997), mid-shaft radial SSI and predicted fracture load (Darling et al. 2012) and tibial cortical vBMD (Pedone et al. 2010), in adults has been shown. In children, a relationship between vitamin D status and tibial cortical bone mass has been found (Sayers et al. 2012). In the only known study assessing associations between 25OHD concentrations and bone geometry in UK dwelling South Asians, vitamin D status was associated with distal radial trabecular density (Darling et al. 2012). These data suggest that vitamin D is associated with positive effects on bone parameters within the radius. However, as the bone parameters that are associated with vitamin D status differ between ethnic groups, it is possible that the mechanisms underlying these associations also differ.

4.1.6 Justification of Study

Despite the fact that the South Asian population are at a greater risk of vitamin D deficiency and have been shown to have lower 25OHD concentrations year round compared to Caucasian populations, data showing bone geometry/associations between 25OHD concentrations and bone geometry in South Asian cohorts are limited. Data thus far has shown that there are differences between South Asian and Caucasian populations, however, further data is needed to see whether previous findings are replicated, before firm conclusions can be drawn.

Determining whether vitamin D$_2$ and vitamin D$_3$ are equally effective in their bone health role is important, as we now know that they are not equipotent at raising 25OHD concentrations. If this has an impact on bone biochemistry that is of detriment to bone health, this would be extremely relevant for the both the prevention, but also the treatment of bone disease such as rickets and osteomalacia, and or optimising bone health throughout the life course. Determining whether there are ethnic differences in bone related biochemical responses to vitamin D$_2$ and vitamin D$_3$ is also
essential, and may identify ethnic differences in the biological effects of vitamin D$_2$ versus vitamin D$_3$ on bone biochemistry.

### 4.1.7 Aims & Hypothesis

**Aim 1** – To examine differences in bone biochemistry (parathyroid hormone (PTH), 1,25(OH)$_2$D) and bone geometry between the South Asian and Caucasian women

*Hypothesis* – PTH concentrations would be higher, and 1,25(OH)$_2$D concentrations would be lower in the South Asian women, compared to the Caucasian women. In the South Asian women, bone size would be smaller, but bone density would be higher, compared to the Caucasian women.

**Aim 2** - To identify associations between serum 25OHD concentrations and markers of bone health (PTH, 1,25(OH)$_2$D, bone density), within and between South Asian and Caucasian women.

*Hypothesis* – In both the South Asian and Caucasian women, 25OHD concentrations would be positively associated with 1,25(OH)$_2$D and bone density, and negatively associated with PTH concentrations.

**Aim 3** - To compare the effects of 15 µg/d (600 IU/d) vitamin D$_2$ vs. vitamin D$_3$ on PTH and 1,25(OH)$_2$D concentrations, in both South Asian and Caucasian women independently.

*Hypothesis* – Both the vitamin D$_2$ and vitamin D$_3$ interventions would lead to a decrease in PTH and an increase in 1,25(OH)$_2$D concentrations, in both ethnic groups, with the vitamin D$_3$ intervention groups showing a greater change.
4.2 METHODS

4.2.1 Participants and Study Protocol

Participants were recruited as part of the D2-D3 Study and followed the study protocol as described in Chapter 2.1. In summary, healthy South Asian and Caucasian women aged 20-65 years were randomised to receive placebo, 15 µg/d vitamin D2 or 15 µg/d vitamin D3, delivered via fortified foods, for a 12-week period and attended three study visits for data collection; week 0, week 6 and week 12.

4.2.2 Relevant Data

The data relevant to bone health, and thus included in these analyses include age, BMI and body fat percentage. The analysis also includes the serum 25OHD, 1,25(OH)₂D, PTH and corrected calcium measures that were taken at all three study visits. In addition, the bone geometry, density and strength data from the pQCT scan at baseline will also be used.

4.2.3 Statistical Analysis

All statistical analyses were performed with the use of SPSS ® software (version 22.0; SPSS Inc., Chicago, IL). Differences were considered to be significant at p<0.05. Data were checked for normality using the Kolmogorov-Smirnov test if sample size was >50 or Shapiro-Wilk otherwise. Comparisons between ethnic groups at baseline were conducted using T-Tests where data were normally distributed, and Mann-Whitney U tests were data were not normally distributed. Partial correlations, controlling for relevant variables, were used to assess relationship between 25OHD concentrations and variables.

To compare bone biochemistry profiles at baseline/week 0, week 6 and week 12 within each treatment group, in South Asian and Caucasian cohorts independently, one-way repeated-measures ANOVA were conducted where data were normally distributed, and Friedman Tests followed by post-hoc Wilcoxon Signed Rank Tests were used where data were not normally distributed.
4.3 RESULTS

4.3.1 Baseline Participant Characteristics

A total of 90 South Asian and 245 Caucasian women were recruited as part of the D2-D3 Study, and included in these analyses.

4.3.1.1 Anthropometrics and Biochemical Bone Parameters

The South Asian cohort were significantly younger age, had a higher BMI and a higher body fat percentage than the Caucasian cohort. The South Asian cohort also had significantly lower 25OHD concentrations than the Caucasian cohort (27.55 nmol/L and 60.28 nmol/L respectively, p<0.001) and significantly higher PTH concentrations than the Caucasian cohort (7.18 pmol/L and 4.04 pmol/L respectively, p<0.001) (Table 4.1).

<table>
<thead>
<tr>
<th>Table 4.1 Anthropometric and bone biochemistry characteristics in the South Asian and Caucasian women</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anthropometrics</strong></td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 245</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 245</td>
</tr>
<tr>
<td>Bodyfat (%)</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 243</td>
</tr>
<tr>
<td><strong>Biochemistry</strong></td>
</tr>
<tr>
<td>Total 25OHD (nmol/L)</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 245</td>
</tr>
<tr>
<td>1,25(OH)(_2)D (pmol/L)</td>
</tr>
<tr>
<td>South Asian 18</td>
</tr>
<tr>
<td>Caucasian 36</td>
</tr>
<tr>
<td>Corrected Calcium (mmol/L)</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 245</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
</tr>
<tr>
<td>South Asian 90</td>
</tr>
<tr>
<td>Caucasian 244</td>
</tr>
</tbody>
</table>

Key: \(^a\) normally distributed analysed by T-test, \(^b\) not-normally distributed analysed by Mann-Witney U
4.3.1.2 Radial Bone Geometry

At the distal (4% site) radius the South Asian cohort had significantly lower total area, but significantly higher total vBMD. At the proximal (66% site) radius, the South Asian cohort had significantly lower cortical area. The SSI of the South Asian cohort was significantly lower than the Caucasian cohort, but the total density T-Score was significantly higher (Table 4.2).

Table 4.2 Descriptive data of radial bone geometry outcomes, in the South Asian and Caucasian women

<table>
<thead>
<tr>
<th></th>
<th>South Asian</th>
<th>Caucasian</th>
<th>T-Test/Mann-W</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td>4% Radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMC (g/cm)</td>
<td>89</td>
<td>1.05</td>
<td>0.16</td>
<td>244</td>
</tr>
<tr>
<td>Total Area (mm²)</td>
<td>89</td>
<td>324.93</td>
<td>56.19</td>
<td>244</td>
</tr>
<tr>
<td>Total Density (mg/cm³)</td>
<td>89</td>
<td>328.64</td>
<td>55.70</td>
<td>244</td>
</tr>
<tr>
<td>Trabecular Density (mg/cm³)</td>
<td>89</td>
<td>189.37</td>
<td>39.87</td>
<td>244</td>
</tr>
<tr>
<td>66% Radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMC (g/cm)</td>
<td>89</td>
<td>1.01</td>
<td>0.35</td>
<td>243</td>
</tr>
<tr>
<td>Total Area (mm²)</td>
<td>89</td>
<td>138.06</td>
<td>68.03</td>
<td>243</td>
</tr>
<tr>
<td>SSI (mm³)</td>
<td>89</td>
<td>230.05</td>
<td>193.15</td>
<td>243</td>
</tr>
<tr>
<td>Cortical Area (mm²)</td>
<td>88</td>
<td>69.92</td>
<td>21.67</td>
<td>243</td>
</tr>
<tr>
<td>Cortical Density (mg/cm³)</td>
<td>88</td>
<td>1123.8</td>
<td>54.53</td>
<td>243</td>
</tr>
<tr>
<td>Cortical:Total Area (%)</td>
<td>88</td>
<td>21.85</td>
<td>6.66</td>
<td>243</td>
</tr>
<tr>
<td>Bone:Muscle Area Ratio (%)</td>
<td>87</td>
<td>6.23</td>
<td>1.46</td>
<td>244</td>
</tr>
<tr>
<td>T-Score Total Density</td>
<td>89</td>
<td>-0.95</td>
<td>0.93</td>
<td>244</td>
</tr>
<tr>
<td>Z-Score Total Density</td>
<td>89</td>
<td>-0.78</td>
<td>0.97</td>
<td>244</td>
</tr>
<tr>
<td>T-Score Trabecular Density</td>
<td>89</td>
<td>-0.33</td>
<td>1.00</td>
<td>244</td>
</tr>
<tr>
<td>Z-Score Trabecular Density</td>
<td>89</td>
<td>-0.23</td>
<td>1.00</td>
<td>244</td>
</tr>
</tbody>
</table>

Key: 4% Radius = distal radius, 66% Radius = mid-shaft radius, BMC = bone mineral content, SSI = strength stain index, <sup>a</sup> normally distributed analysed by T-test, <sup>b</sup> not-normally distributed analysed by Mann-Witney U
4.3.2 Associations Between 25OHD and Bone Profile

There was a significant positive correlation between 25OHD concentrations and 1,25(OH)D concentrations in the South Asian women, but not the Caucasian women (Figure 4-2). Calcium was significantly positively correlated (Figure 4-3), and PTH was negatively correlated (Figure 4-4), with 25OHD in both the South Asian and Caucasian women. There were no significant correlations between 25OHD concentrations and any of the outcomes from the pQCT scans, in either ethnic group (Table 4.3).

<table>
<thead>
<tr>
<th></th>
<th>South Asian r value</th>
<th>Caucasian r value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)D (pmol/L)</td>
<td>0.632*</td>
<td>-0.076</td>
</tr>
<tr>
<td>Corrected Calcium (mmol/L)</td>
<td>0.237*</td>
<td>0.231***</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>-0.299**</td>
<td>-0.259***</td>
</tr>
<tr>
<td>4% BMC (g/cm)</td>
<td>-0.147</td>
<td>0.032</td>
</tr>
<tr>
<td>4% Total Area (mm²)</td>
<td>-0.057</td>
<td>-0.044</td>
</tr>
<tr>
<td>4% Total Density (mg/cm³)</td>
<td>-0.041</td>
<td>0.015</td>
</tr>
<tr>
<td>4% Trabecular Density (mg/cm³)</td>
<td>-0.005</td>
<td>0.095</td>
</tr>
<tr>
<td>66% BMC (g/cm)</td>
<td>-0.054</td>
<td>0.034</td>
</tr>
<tr>
<td>66% Total Area (mm²)</td>
<td>-0.011</td>
<td>0.007</td>
</tr>
<tr>
<td>SSI (mm³)</td>
<td>-0.081</td>
<td>-0.013</td>
</tr>
<tr>
<td>66% Cortical Area (mm²)</td>
<td>-0.108</td>
<td>0.047</td>
</tr>
<tr>
<td>66% Cortical Density (mg/cm³)</td>
<td>-0.103</td>
<td>-0.004</td>
</tr>
<tr>
<td>Cortical:Total Area (%)</td>
<td>-0.053</td>
<td>0.044</td>
</tr>
<tr>
<td>Bone:Muscle Area Ratio (%)</td>
<td>0.088</td>
<td>-0.043</td>
</tr>
<tr>
<td>T-Score Total Density</td>
<td>-0.042</td>
<td>0.013</td>
</tr>
<tr>
<td>Z-Score Total Density</td>
<td>-0.039</td>
<td>0.042</td>
</tr>
<tr>
<td>T-Score Trabecular Density</td>
<td>0</td>
<td>0.095</td>
</tr>
<tr>
<td>Z-Score Trabecular Density</td>
<td>0.002</td>
<td>0.107</td>
</tr>
</tbody>
</table>

Key: PTH = parathyroid hormone, 4% Radius = distal radius, 66% Radius = mid-shaft radius, BMC = bone mineral content, SSI = strength stain index, ***p<0.001, **p<0.01, *p<0.05
Figure 4-2 Scatterplots of \(1,25(OH)_2D\) against \(25OHD\) in South Asian and Caucasian women
Partial correlation, controlling for age, BMI and body fat percentage in South Asian \((r=0.623, p=0.011)\) and Caucasian \((r=-0.076, p=0.676)\) women.

Figure 4-3 Scatterplots of corrected calcium against \(25OHD\) in South Asian and Caucasian women
Partial correlation, controlling for age, BMI and body fat percentage in South Asian \((r=0.237, p=0.027)\) and Caucasian \((r=0.231, p<0.001)\) women.

Figure 4-4 Scatterplots of PTH concentrations against \(25OHD\) in South Asian and Caucasian women
Partial correlation, controlling for age, BMI and body fat percentage in South Asian \((r=-0.299, p=0.005)\) and Caucasian \((r=-0.259, p<0.001)\) women.
### 4.3.3 Changes in Biochemical Bone Parameters

#### 4.3.3.1 At Baseline (Week 0)

There were no significant differences in any of the anthropometric or bone biochemistry measures between treatment groups, within either ethnic group, except for 1,25(OH)2D, which showed a significant difference between the three South Asian intervention groups (p=0.050), although in post-hoc analyses the closest to significance was between the placebo and vitamin D3 intervention (p=0.055) (Table 4.4). When comparing between ethnic groups, BMI in the South Asian vitamin D2 intervention group was significantly higher than both the Caucasian vitamin D2 and D3 group (p=0.030 and p=0.007, respectively). Both the South Asian placebo and vitamin D3 intervention groups had significantly higher waist:hip ratios (WHR) than all three Caucasian intervention groups (p<0.040 in all cases), and the South Asian vitamin D2 group had a significantly higher WHR than the Caucasian vitamin D3 intervention group (p=0.019). Body fat percentage was significantly higher in the South Asian vitamin D2 group than all three Caucasian intervention groups (p<0.028 in all cases). Serum 25OHD concentrations were consistently significantly lower in the South Asian cohort than the Caucasian cohort (SA: 30.8, 29.9 and 23.9 nmol/L, Cauc: 58.8, 61.0 and 60.3 nmol/L, p<0.001). There were no differences between 1,25(OH)2D or calcium concentrations between ethnic groups, however PTH concentrations were consistently significantly higher in the South Asian cohort than the Caucasian cohort (SA: 7.27, 7.10 and 7.20 pmol/L, Cauc: 3.96, 4.16 and 3.98 pmol/L, p<0.001).
### Table 4.4 Anthropometric and bone biochemistry profile of the South Asian and Caucasian women, within the three intervention groups, across the three study visits

<table>
<thead>
<tr>
<th></th>
<th>Week 0 / Visit 1</th>
<th></th>
<th>Week 6 / Visit 2</th>
<th></th>
<th>Week 12 / Visit 3</th>
<th></th>
<th>Repeated-Measures ANOVA</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-value 0.652 0.235</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
<td>p-value 0.751 0.286</td>
</tr>
<tr>
<td>Waist:Hip Ratio</td>
<td>Placebo 17 0.85 [0.07] 47 0.79 [0.07]</td>
<td>Vit D2 35 0.83 [0.08] 98 0.80 [0.07]</td>
<td>Vit D3 38 0.85 [0.09] 99 0.78 [0.07]</td>
<td>p-value 0.406 0.323</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
</tr>
<tr>
<td></td>
<td>Placebo 17 0.85 [0.07] 47 0.79 [0.07]</td>
<td>Vit D2 35 0.83 [0.08] 98 0.80 [0.07]</td>
<td>Vit D3 38 0.85 [0.09] 99 0.78 [0.07]</td>
<td>p-value 0.406 0.323</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
<td>p-value 0.150 0.737</td>
</tr>
<tr>
<td>% Body Fat</td>
<td>Placebo 17 33.08 [6.46] 46 29.02 [6.76]</td>
<td>Vit D2 35 33.51 [5.16] 98 29.16 [5.83]</td>
<td>Vit D3 38 31.65 [8.50] 98 28.82 [6.61]</td>
<td>p-value 0.505 0.932</td>
<td>p-value 0.448 0.742</td>
<td>p-value 0.448 0.742</td>
<td>p-value 0.448 0.742</td>
<td>p-value 0.448 0.742</td>
</tr>
<tr>
<td>25OHD (nmol/L)</td>
<td>Placebo 17 30.77 [27.2] 48 58.80 [23.1]</td>
<td>Vit D2 35 29.94 [23.1] 98 61.04 [29.4]</td>
<td>Vit D3 38 23.90 [18.9] 99 60.25 [25.3]</td>
<td>p-value 0.415 &lt;0.001</td>
<td>p-value 0.415 &lt;0.001</td>
<td>p-value 0.415 &lt;0.001</td>
<td>p-value 0.415 &lt;0.001</td>
<td>p-value 0.415 &lt;0.001</td>
</tr>
<tr>
<td>1,25(OH)₂D (pmol/L)</td>
<td>Placebo 6 56.90 [14.2] 12 44.94 [12.3]</td>
<td>Vit D2 6 41.00 [8.9] 12 46.16 [13.9]</td>
<td>Vit D3 6 37.37 [15.8] 12 45.76 [14.0]</td>
<td>p-value 0.050 0.975</td>
<td>p-value 0.050 0.975</td>
<td>p-value 0.050 0.975</td>
<td>p-value 0.050 0.975</td>
<td>p-value 0.050 0.975</td>
</tr>
<tr>
<td>Corrected Calcium (nmol/L)</td>
<td>Placebo 17 2.30 [0.08] 48 2.31 [0.09]</td>
<td>Vit D2 35 2.32 [0.06] 98 2.33 [0.08]</td>
<td>Vit D3 38 2.32 [0.08] 99 2.33 [0.09]</td>
<td>p-value 0.623 0.521</td>
<td>p-value 0.980 0.672</td>
<td>p-value 0.980 0.672</td>
<td>p-value 0.980 0.672</td>
<td>p-value 0.980 0.672</td>
</tr>
<tr>
<td>PTH (pmol/L)</td>
<td>Placebo 17 7.27 [2.88] 48 3.96 [1.51]</td>
<td>Vit D2 35 7.10 [2.85] 98 4.16 [1.74]</td>
<td>Vit D3 38 7.20 [3.21] 98 3.98 [1.68]</td>
<td>p-value 0.980 0.690</td>
<td>p-value 0.249 0.271</td>
<td>p-value 0.249 0.271</td>
<td>p-value 0.249 0.271</td>
<td>p-value 0.249 0.271</td>
</tr>
</tbody>
</table>

M = mean, SD = standard deviation, *normally distributed analysed by repeated-measures ANOVA, †not-normally distributed analysed by Friedman Test with post-hoc Wilcoxon Signed Rank Test (sig adjusted to p-value <0.017), ‡not-normally distributed analysed by Wilcoxon Signed Rank Test §significantly different from baseline/week 0 ¶significantly different from week 6
4.3.3.2 Changes in Anthropometric Measurements

There was a significant change in BMI over time in both the Caucasian vitamin D$_2$ and vitamin D$_3$ groups. The vitamin D$_2$ group showed a significantly higher BMI at week 6 and week 12 compared to baseline/week 0. Although the vitamin D$_3$ group demonstrated a significant change over time, post-hoc analyses showed no significant differences between intervention groups to the adjusted significance levels of $p<0.017$. There was also a significant change in body fat percentage over time in both the South Asian and Caucasian vitamin D$_3$ groups. The Caucasian group showed a significantly lower BMI at week 6 compared to baseline. Again, within the South Asian cohort there were no significant differences between visits in post-hoc analyses.

4.3.3.3 Changes in 25OHD Concentrations

As expected, and presented in Chapter 3.3.8.1, there was a significant effect of the treatment over time on 25OHD concentrations (Figure 3-3). Both the South Asian and Caucasian placebo groups saw a significant decrease in 25OHD over time, with 25OHD concentrations at week 6 and week 12 significantly lower than at baseline for both ethnic groups ($p<0.001$ in all cases), and week 12 significantly higher than week 6 within the Caucasian ($p<0.001$). Whereas the vitamin D$_2$ and D$_3$ intervention in both ethnic groups showed a significant increase in 25OHD over time, with both ethnic groups on the vitamin D$_2$ and vitamin D$_3$ intervention showing significantly higher 25OHD concentrations at week 6 and 12 compared with baseline ($p<0.001$ in all cases), and the vitamin D$_3$ intervention for both ethnic groups also showed significantly higher 25OHD concentrations at week 12 compared to week 6 (SA $p=0.033$, Cauc $p<0.001$).
4.3.3.4 Changes in 1,25(OH)$_2$D Concentrations

For both the South Asian and Caucasian cohorts, there was a significant effect of treatment over time on 1,25(OH)$_2$D concentrations in the vitamin D$_2$ intervention, with 1,25(OH)$_2$D significantly higher at week 12 compared with baseline for both ethnic groups (p=0.028 in both cases). The significant relationships detailed in Table 4.4 are presented graphically in Figure 4-5.

Figure 4-5 1,25(OH)$_2$D concentrations [mean +SEM] at baseline (wk 0) and week 12, within intervention groups split by ethnicity
*Indicates significant difference between week 0 and week 12 (p=0.028)
### 4.3.3.5 Changes in Calcium Concentrations

There was a significant effect of treatment over time on serum corrected calcium concentrations in all intervention groups, in both the South Asian and Caucasian cohorts. The significant relationships detailed in Table 4.4 are presented graphically in Figure 4-6. All groups, with the exception of the South Asian placebo cohort, showed a significantly lower serum calcium concentration at week 6 compared to baseline, and all groups showed significantly higher calcium concentrations at week 12 compared to both baseline and week 6.

![Corrected Calcium Concentrations](image)

*Figure 4-6 Corrected calcium concentrations [mean +SEM] at week 0, 6 and 12, within intervention groups split by ethnicity
*indicates significantly higher concentrations at week 12, compared to both week 0 and week 6 (p<0.05)
4.3.3.6 Changes in PTH Concentrations

There was a significant effect of treatment over time across the three study visits within the Caucasian placebo group, Caucasian vitamin D$_2$ group and South Asian vitamin D$_3$ group. Both Caucasian groups showed significantly higher PTH concentrations at week 6 and week 12 compared to baseline, whereas the South Asian vitamin D$_3$ group showed a significantly lower PTH concentration at week 12 compared to both baseline and week 6. The significant relationships detailed in Table 4.4 are presented graphically in Figure 4-7.

Figure 4-7 PTH concentrations [Mean +SEM] at weeks 0, 6 and 12, within intervention groups split by ethnicity
*indicates significant difference at week 12 compared to week 0 (p<0.05)
4.4 DISCUSSION

The aims of this study were to compare bone biochemistry and bone geometry between South Asian and Caucasian women, and to then assess whether there were associations between these measures and 25OHD concentrations. The study further aimed to assess changes in bone biochemistry in response to a 12-week randomised-controlled trial giving placebo, vitamin D$_2$ or vitamin D$_3$ in both South Asian and Caucasian women.

4.4.1 Bone Biochemistry between Ethnic Groups

The South Asian women had lower 25OHD concentrations compared to the Caucasian women, and significantly higher PTH concentrations. The mean serum 25OHD for the South Asian women was within the ‘insufficient’ range of 25-50 nmol/L, but close to the ‘deficiency’ range of <25 nmol/L at 27.6 nmol/L, whereas the mean serum 25OHD for the Caucasian women was within the ‘sufficiency’ range of 50-75 nmol/L at 60.3 nmol/L. The mean PTH concentrations were within the ‘normal’ reference ranges of 1.5-7.6 pmol/L, however again the South Asian women were close to the higher end of the normal range with a mean of 7.3 pmol/L which would be detrimental to bone health in the long term. Several studies have shown an inverse relationship between PTH and serum 25OHD at 25OHD concentrations <75 nmol/L (Chapuy et al. 1997, Lips et al. 1988) and therefore the higher PTH concentrations in the South Asian women would be expected with the lower 25OHD concentrations seen.

There was no difference in 1,25(OH)$_2$D concentrations between the ethnic groups (although it is important to note that sample size was very small), nor corrected calcium concentrations. Serum calcium concentrations were within the reference ranges of 2.1-2.6 mmol/L, giving no indication of hyperparathyroidism in either group, and therefore the higher PTH concentrations seen in the South Asian women are likely to be driven by the lower 25OHD concentrations and thus the process by which calcium and phosphorus concentrations are regulated.

4.4.2 Bone Geometry between Ethnic Groups

The South Asian women had a smaller bone size, but increased total bone density at the distal (4%) radius compared to the Caucasian women, and they also had lower cortical bone size at the mid-shaft (66%) radius, and lower radial bone strength (SSI). The finding of smaller radial bone size at the distal (4%) radius concurs with findings in post-menopausal South Asian women (Darling et al. 2013b), younger South Asian women (Ward et al. 2007) and even with other Asian groups (Walker et
The finding of increased total volumetric bone density also concurs with previous research (Darling et al. 2013b). However, bone size has not consistently been shown to be lower in South Asian women. A study of pre-menopausal women of Bangladeshi origin in Finland showed no difference in bone size, total bone mineral content or trabecular density at the distal (4%) radius, compared to Finnish Caucasians (Islam et al. 2012). This supports our finding of no ethnic differences in bone mineral content and trabecular density, but contrasts with our results on bone size at the distal (4%) radius. At the mid-shaft (66%) radius, Islam et al. (2012) found that the Bangladeshi women had smaller bone size and cortical size, and lower total bone mineral content and cortical density than the Caucasian women, however, the bone strength (SSI) was similar. Darling and colleagues (2013b) also found a smaller bone size at the mid-shaft radius, but showed lower radial bone strength (SSI) in the South Asian women. The data from Islam and colleagues (2012) supports our finding of smaller cortical size, but our study did not show ethnic differences in total bone mineral content or cortical density at the mid-shaft radius. It also does not concur with our findings of lower radial bone strength (SSI) in the South Asian cohort, which do concur with the findings by Darling and colleagues (2013b).

The most likely explanation for the differences between our study results with previous research, is age and menopausal status. Previous studies have only been in post-menopausal women (Darling et al. 2013b), younger pre-menopausal women aged 20-48 years (Islam et al. 2012), or 18-36 years (Ward et al. 2007), whereas the current study had both pre- and post-menopausal women with an age range of 20-65 years.

The SSI of the participants in this study cohort compared with Darling et al. (2013b) was 230mm$^3$ and 196mm$^3$ respectively in the South Asian women, and 245mm$^3$ and 238mm$^3$ respectively in the Caucasian women. Both the South Asian and Caucasian women in our cohort therefore had higher SSI than Darling and colleagues study (2013b). This may also be down to the age and menopausal status of the women recruited into these studies, as we recruited women aged 20-65 years and therefore had both pre- and post-menopausal women in the study whereas Darling and colleagues recruited just post-menopausal women.

4.4.3 Associations between Bone Biochemistry and 25OHD Concentrations

In both the South Asian and Caucasian women, there was a positive association between serum total 25OHD concentrations and serum (corrected) calcium concentrations. There was also a negative association between 25OHD and PTH concentrations, which is consistent with previous findings.
(Need et al. 2000). In the South Asian women, there was also a positive association between 25OHD and 1,25(OH)_{2}D concentrations, which is consistent with previous findings of a concentration dependent variation in 1,25(OH)_{2}D with 25OHD (Rejnmark et al. 2008).

**4.4.4 Associations between Radial pQCT Outcomes and 25OHD Concentrations**

There were no associations between any of the radial pQCT measures with 25OHD concentrations in either ethnic group, which contrasts with previous studies findings. A study in older women found that total bone density of the ultra-distal radius was associated with 25OHD concentrations (Boonen et al. 1997) and a study in men has shown a trend towards an association between 25OHD and cortical BMD (Viljakainen et al. 2009). Of most relevance is a study that completed a radial pQCT scan in 50 Caucasian and 18 South Asian post-menopausal women (Darling et al. 2012). They found an association between 25OHD concentrations and the distal radial trabecular density in the South Asian women, and associations between 25OHD concentrations and BMC and bone size at the distal radius, and BMC and bone strength (SSI) at the mid-shaft radius in the Caucasian women.

It is not surprising to find no association of 25OHD concentrations with bone size, as bone size is determined in childhood during growth. However, it is unclear why our data did not show any association between 25OHD concentrations and any of the other outcomes from the pQCT scan, particularly bone density. It would make biological sense that 25OHD concentrations would be beneficially associated with bone mineralization and therefore bone density, and a large population based study has shown a positive association between 25OHD concentrations and BMD in younger and older adults (Bischoff-Ferrari et al. 2004). One possible explanation for the lack of associations seen in this study could be due to the narrow range of 25OHD concentrations seen in the participants included within the study, compared with previous studies (Darling et al. 2012) (95% confidence interval lower and upper means were 22.89 to 32.21 nmol/L in the South Asian women and 56.94-63.62 nmol/L in the Caucasian women). In the screening process, anyone who had been taking vitamin D supplements, had been on a sun holiday, or had been on a sunbed within the three months prior to the study were not recruited onto the study. In contrast, previous studies (Darling et al. 2012) did not exclude vitamin D supplementation use and thus had higher and more variable 25OHD concentrations (95% confidence interval lower and upper means of 47.0 to 67.7 nmol/L in their South Asian cohort and 79.2 to 90.4 nmol/L in their Caucasian cohort (personal communication)).
4.4.5 Effect of Vitamin D2 and D3 Supplementation on Bone Parameters

As expected, 25OHD concentrations decreased in both ethnic cohorts on the placebo intervention as a result of the season, and increased in both ethnic cohorts on the vitamin D$_2$ and vitamin D$_3$ interventions. As discussed in 3.3.8, supplementation with vitamin D$_3$ led to a greater increase in 25OHD concentrations than vitamin D$_2$, in both South Asian and Caucasian ethnic groups.

Corrected calcium concentrations increased after 12-weeks of either placebo, vitamin D$_2$ or vitamin D$_3$ in both the South Asian and Caucasian cohort. As calcium concentrations are tightly regulated the measurement of corrected calcium was not aimed at examining the response to the intervention, but to ensure that other changes seen were not a result of hyperparathyroidism. The lowest and highest mean corrected calcium concentration seen across all three visits was 2.23 to 2.39 mmol/L, within the reference range of 2.1-2.6 mmol/L, therefore giving no indication of hypercalcaemia.

Both the South Asian and Caucasian women in the vitamin D$_2$ intervention saw a significant increase in 1,25(OH)$_2$D, but the vitamin D$_3$ intervention group did not, despite having a greater change in 25OHD concentrations and previous studies showing a slight increase in 1,25(OH)$_2$D following vitamin D$_3$ supplementation (Lips et al. 1988). Study numbers may have been a limiting factor in this case, as there were only 18 South Asians and 36 Caucasians whose 1,25(OH)$_2$D concentrations were measured. Despite there only being significant differences within the vitamin D$_2$ intervention groups, the mean 1,25(OH)$_2$D at baseline and week 12 showed a direction of change towards a decrease in 1,25(OH)$_2$D in the placebo groups for both ethnic groups, and a direction of change towards an increase in both the vitamin D$_2$ and vitamin D$_3$ interventions in both ethnic groups. Although significant changes in 1,25(OH)$_2$D were not shown in all of the vitamin D intervention groups, previous evidence suggest that calcium absorption efficiency can be improved by increasing 25OHD concentrations, even without seeing any changes in 1,25(OH)$_2$D concentrations (Heaney et al. 1997).

Of great interest were the differences seen in the PTH concentrations. In the Caucasian cohort, both the placebo and vitamin D$_2$ groups showed a significant increase in PTH concentrations over the 12-week intervention, from 3.96 to 4.59 pmol/L in the placebo group and 4.16 to 4.62 pmol/L in the vitamin D$_2$ group, respectively. However, there was no change in PTH concentration in the vitamin D$_3$ group (3.98 to 3.94 pmol/L), which suggests that the vitamin D$_3$ intervention prevented the increase in PTH that would usually be seen as a result of season in the Caucasian cohort (represented by the placebo group). Of particular note was the finding that the vitamin D$_2$ intervention did not blunt the increase in PTH. In the South Asian cohort, neither the placebo intervention nor the vitamin D$_2$
intervention showed any significant change in PTH concentrations, whereas the vitamin D₃ intervention led to a significant decrease in PTH concentrations, from 7.20 to 4.75 pmol/L. At the end of the intervention, the placebo and vitamin D₂ intervention groups continued to have higher PTH concentrations, at 6.90 and 5.56 pmol/L, respectively. These data suggest that the vitamin D₃ intervention has a beneficial effect on PTH concentrations, but the vitamin D₂ intervention does not.

The findings across both South Asian and Caucasian cohorts shows the beneficial impact of the vitamin D₃ intervention, over the vitamin D₂ intervention, on PTH which would in turn influence bone turnover markers. The likely explanation for seeing an increase in PTH concentrations in the Caucasian placebo and vitamin D₂ groups, but a decrease in PTH concentrations in the South Asian vitamin D₃ group, is the baseline PTH concentration. The Caucasian participants had PTH concentrations within the normal range at baseline, and therefore seeing a beneficial reduction in PTH concentrations were not likely or necessary from a bone health perspective. In contrast, the South Asian women (representative of subjects with a low vitamin D status) started with higher PTH concentrations, and so there was a greater opportunity to beneficially reduce PTH concentrations.

Vitamin D₃ supplementation has been shown to reduce PTH concentrations by 15% in previous supplementation trials (Lips et al. 1988), and 29% in food fortification trials (Bonjour et al. 2013). However a similar study, comparing the effectiveness of vitamin D₂ compared with vitamin D₃ on serum 25OHD, has shown no intervention-related changes in PTH concentrations among the intervention groups (Logan et al. 2013). However, the change in 25OHD concentrations within the placebo, vitamin D₂ and vitamin D₃ intervention groups in the study by Logan and colleagues were -44nmol/L, -18nmol/L and 0nmol/L, respectively, whereas in the present study both the vitamin D₂ and vitamin D₃ interventions in both ethnic groups resulted in a mean increase in 25OHD concentrations.

4.4.6 Limitations

There are some limitations to this work that should be considered. Both our Caucasian and South Asian women were in relatively good health and were also of relatively high socio-economic status, and so may not be representative of the bone health of the wider population. It is likely that this was the reason for not replicating previous findings of other studies.

The study was also primarily designed to investigate changes in serum 25OHD concentrations in response to placebo, vitamin D₂ or vitamin D₃, and therefore the study length was not designed to
study the effects of vitamin supplementation on direct changes to bone (i.e. vBMD); therefore biochemical markers were used to assess the effect on bone through calcium and phosphorus regulation. Due to funding limitations, we were not able to measure bone turnover markers, however we have kept samples for future analyses and this will be a key area for further research.

4.4.7 Concluding Remarks and Areas for Further Research

To conclude, these data showed that South Asian women have smaller bones, yet have structural adaptations to improve strength such as increased total density at the distal radius. Despite this adaptation, the negative impact of the smaller bone size prevails, and South Asian women are still likely to be at greater risk of fracture than Caucasian women of the same age.

Unlike previous studies, we did not see any association between 25OHD concentrations and bone size, mass, density or strength. However, compared with previous studies that have shown associations, our study had a narrow range of 25OHD concentrations and therefore differences in bone associated with 25OHD concentrations were less likely to be identified in this cohort of South Asian and Caucasian women.

Finally, in response to supplementation with vitamin D$_2$ and vitamin D$_3$, the most interesting and clinically relevant finding of this study was the beneficial effect of vitamin D$_3$, over vitamin D$_2$ and the placebo interventions, on PTH concentrations in both the South Asian and Caucasian cohorts. In the South Asian cohort, who had a much higher PTH concentrations than the Caucasian cohort at baseline, the vitamin D$_3$ intervention led to a significant decrease in PTH. This is extremely important as it shows that in a clinical setting, the use of vitamin D$_3$ may be preferential, over vitamin D$_2$, for improving PTH concentrations and in turn benefitting bone health. As shown in the Caucasian cohort, vitamin D$_2$ does not just have a beneficial effect on PTH, over vitamin D$_2$, when PTH concentrations are higher, as the vitamin D$_3$ intervention was able to maintain PTH concentrations when the placebo and vitamin D$_2$ intervention groups showed an increase in PTH.

Further work will involve measuring bone turnover markers (CTX) in this cohort to assess response to supplementation. It would be anticipated that they would change in line with the differences shown in PTH concentrations. It would also be interesting to split the cohort by menopausal status, to see whether previous findings in post-menopausal women can be replicated, particularly in respect to bone geometry and associations with 25OHD concentrations. There is also further opportunity to explore whether current bone health/bone status affects response to vitamin D supplementation, as shown previously (Appendix 1).
Chapter 5 - THE D2-D3 CVD STUDY
5.1 INTRODUCTION

5.1.1 CVD Incidence

Cardiovascular disease (CVD) is a broad term used to describe any disease that involves the heart or blood vessels, the most common being coronary heart disease and stroke. Since 1961, cardiovascular diseases have been the leading cause of death in the UK. However, data collected from 2012 showed that it is now the second main cause of death, with cancer causing 29% of deaths and CVD causing 28% (n161,252) of deaths respectively (Townsend et al. 2014). Across the world, the estimated number of deaths from CVD in 2012 was 17.5 million (WHO Global Health Observatory).

Coronary heart disease (CHD) is the most common form of CVD, causing 46% of all deaths from CVD in 2012, and the two main forms of CHD are heart attack/myocardial infarction and angina. Strokes, blockage of blood vessels supplying the brain, were the cause of 28% of all deaths from CVD in 2012 (Townsend et al. 2014).

A gradual build-up of fatty material, known as atherosclerotic deposits, atheroma or plaques, on the walls of blood vessels are the main cause of CVD, with the resultant narrowing and hardening of the coronary arteries predisposing individuals to CHD, and narrowing and hardening of blood vessels supplying the brain predisposing individuals to a stroke. Having high or undesirable blood lipids, the thresholds for which are shown below, has been associated with a higher risk of atherosclerosis (Nicholls et al. 2011).

5.1.2 CVD Risk Factors

There are both non-modifiable and modifiable risk factors of CVD. The non-modifiable factors include age, sex, family history, genetics and birth weight. The modifiable factors include diet, smoking, lack of exercise, diabetes and glucose intolerance, being overweight or obese, oral contraceptive use, high blood pressure, and an undesirable lipid profile (Warburton et al. 2007; Nicholls et al. 2011; Poulter 2003). The undesirable lipid profile associated with an increased risk of CVD includes high triglycerides (>2 mmol/L), high total cholesterol (>5 mmol/L), high low-density-lipoprotein (LDL) cholesterol concentrations (>3 mmol/L), and low high-density lipoprotein (HDL) cholesterol concentrations (<1 mmol/L) (NHSGGC 2015).
5.1.3 Vitamin D and CVD

5.1.3.1 Evidence of Associations

Epidemiological studies have documented deficient (<25 nmol/l) or insufficient (<50 nmol/l) 25OHD concentrations in patients with myocardial infarction (MI) (Scragg et al. 1990) and stroke (Poole et al. 2006). Cross-sectional and case-control studies have indicated that increasing 25OHD concentrations may have a role in risk reduction of CVD (Hintzpeter et al. 2008; Kendrick et al. 2009; Scragg et al. 1990). A systematic review and meta-analysis of prospective studies evaluating the prognostic value of 25OHD for CVD incidence and mortality found an inverse association between 25OHD concentrations and CVD risk, with five out of nine studies reporting a significant increase in risk in subjects with lower 25OHD concentrations (Grandi et al. 2010). Associations between 25OHD concentrations and CVD have also been shown in large longitudinal studies. Wang and colleagues (2008) followed 1,739 participants from the Framingham Offspring Study over 5.4 years and concluded that vitamin D deficiency was associated with incident cardiovascular disease, after finding that individuals with 25OHD concentrations <15 ng/ml (<37.5 nmol/l) had a hazard ratio of 1.62 for incident cardiovascular events compared to those with 25OHD concentrations >15 ng/ml (>37.5 nmol/l). Ginde and colleagues (2009) followed 3,480 participants from the NHANES III cohort over 7.3 years and also found that 25OHD concentrations were inversely associated with CVD rate. Also using the NHANES III cohort, Kendrick and colleagues (2009) conducted cross-sectional analyses on 16,603 to examine the association between 25OHD concentrations and prevalence of CVD. They found that participants with 25OHD concentrations <20 ng/ml (<50 nmol/L) had an increased risk of prevalent CVD than those with 25OHD >20 ng/ml (>50 nmol/L) with an odds ratio of 1.20. In all three of these large longitudinal studies, the radioimmunoassay kit (RIA, DiaSorin) was used to measure 25OHD concentrations.

5.1.3.2 Mechanism of Action

There is evidence to support the biological plausibility of a relationship between 25OHD concentrations and CVD, via both direct and indirect mechanisms. When considering the direct mechanisms by which 25OHD may modify CVD risk there are several hypothesized mechanisms (Norman and Powell 2014; Garcia and Martini 2010), and although the precise mechanisms are unclear, effects on CVD via the renin-angiotensin system and vascular calcification are two possibilities. The mechanisms that are proposed involve the presence of the vitamin D receptor (VDR) and its potential modulation on gene expression. It is now recognized that the VDR is present in a variety of tissues and cells throughout the body, including cardiomyocytes, vascular smooth
muscle and endothelium. The active form of vitamin D, 1-25(OH)₂D, binds to the VDR, which then may influence the activity of cells and gene activity.

It has, however, been suggested that 25OHD indirectly modifies risk of CVD by its association with cardiovascular risk factors such as diabetes (Martins et al. 2007; Matilla et al. 2007), obesity (Aasheim et al. 2008; Martins et al. 2007), hypertension (Forman et al. 2007; Burgaz et al. 2011), smoking (Brot et al. 1999; Hill et al. 2006), or cholesterol concentration (Auwerx et al. 1992; Scragg et al. 1995). 25OHD concentrations are also inversely associated with PTH concentrations, and PTH concentrations are associated with an increased risk of cardiovascular events and mortality (Pilz et al. 2009). Mechanistically this may be down to the PTH increasing blood pressure and exerting effects on the heart, including myocardial hypertrophy and pro-arrhythmic actions (Fitzpatrick et al. 2008).

5.1.3.3 Supplementation with Vitamin D

Despite the evidence from observational studies suggesting associations between 25OHD concentrations and CVD, controlled intervention studies are required to determine the causative relationship between vitamin D and CVD risk. So far it is unclear whether vitamin D intervention studies affect cardiovascular outcomes, due to the lack of consensus between study findings. CVD was not included as a health outcome in the recent SACN Report on New Dietary Requirements for Vitamin D (SACN 2015) or indeed in the IOM Vitamin D recommendations, due to lack of randomised-controlled trial data.

In the past 5 years there have been a number of systematic reviews and meta-analyses of randomised controlled trials (RCTs) that have CVD as secondary outcomes, following vitamin D supplementation. A systematic review of eight randomised trials, with CVD as a secondary outcome, reported no significant reduction in CVD risk with vitamin D supplementation of approximately 25 µg (1000 IU), calcium supplementation or a combination of vitamin D plus calcium supplementation (Wang et al. 2010). Similarly, another systematic review and meta-analyses of 51 RCTs concluded that the trial data available to date were not able to demonstrate a statistically significant reduction in CVD risk with vitamin D supplementation (Elamin et al. 2011). In a systematic review of 21 RCTs (n=13,033; mean/median age ≥ 60 years; ≥ 1 year follow-up) assessing the effects of vitamin D compared with placebo reported that estimated hazard ratios (95% CIs) were not significant for cardiac failure (0.82 (0.58-1.15)), myocardial infarction (0.96 (0.83-1.10)) and stroke (1.07 (0.91-1.29)) (Ford et al. 2014). Similar results were reported from a meta-analysis of 3 placebo-controlled trials which reported no significant reduction in myocardial infarction or stroke (Bolland et al. 2011).
A significant decrease in triglycerides and PTH concentrations were shown following a year of supplementation with 83 μg/d vitamin D₃ (Zitterman et al. 2009). Furthermore, a meta-analysis of 12 RCTs that evaluated the effects of vitamin D supplementation on blood lipid concentrations, showed a statistically significant pooled mean net change of 3.23 mg/dl in LDL-C concentration in the vitamin D supplementation group compared with placebo, yet no significance in corresponding total cholesterol, HDL-C and triglyceride concentrations (Wang et al. 2012). Further meta-analyses of RCTs have shown that vitamin D supplementation significantly lowers systolic blood pressure by -2.44 mmHg (Wu et al. 2010), and diastolic blood pressure by -3.1 mmHg (Witham et al. 2009).

5.1.4 Current Consensus on Vitamin D and CVD

In 2010, when the IOM updated recommendations for vitamin D in the US, they concluded that the evidence that vitamin D prevents CVD, or other cardiometabolic outcomes were inconsistent and inconclusive and did not meet criteria for establishing a cause and effect relationship (IOM, 2011). In the UK, the SACN Vitamin D Working Group Draft Vitamin D and Health Report have re-examined the data since the IOM publication and have concluded that the data on vitamin D and non-musculoskeletal health outcomes, including CVD, remained insufficient at this time to inform the setting of dietary reference values (DRVs) for vitamin D (SACN 2015).

5.1.5 Justification of Study

Studies of migrant South Asian populations have shown an increased risk of cardiovascular death compared with other ethnic groups (McKeigue et al. 1989; Harding 2003). A recent review has warned of the higher risk of obesity, diabetes and coronary heart disease among Asian Indians, primarily due to poor vitamin D status and the resulting up-regulation of PTH concentrations (McCarthy 2009). South Asian cohorts are therefore a target group for interventions or treatments that would improve both vitamin D status and CVD risk.

Since the IOM in the US set their vitamin D recommendation of 15 μg/d in 2010 (IOM 2011), and the UK soon looks set to recommend 10 μg/d (although slightly lower) (SACN 2015), determining whether this daily intake of vitamin D has any beneficial effect on markers of cardiovascular health is of interest, particularly within the high risk South Asians. Determining whether vitamin D₂ and vitamin D₃ lead to equal effects on any changes in cardiovascular health has not been investigated previously. Since one of the hypothesized mechanisms of vitamin D on CVD risk is via the action of the VDR on gene expression, and the VDR is thought to have a higher affinity for vitamin D₃ over vitamin D₂ (Houghton & Vieth 2006), it is plausible that vitamin D₂ and D₃ will exert different effects on CVD risk.
5.1.6 Aims & Objectives

**Aim 1** - To examine differences in CVD risk (anthropometrics, blood pressure, blood lipids) between the South Asian and Caucasian women.

**Hypothesis** – South Asian women would have higher risk factors for CVD than the Caucasian women

**Aim 2** – To identify associations between serum 25OHD and blood lipid concentrations, between the South Asian and Caucasian women.

**Hypothesis** – In both the South Asian and Caucasian women, serum 25OHD would be negatively associated with triglyceride, total cholesterol and LDL cholesterol concentrations, and positively associated with HDL cholesterol concentrations

**Aim 3** - To examine the effects of 15 µg/d (600 IU/d) vitamin D$_2$ vs. vitamin D$_3$ on blood lipid concentrations, in both South Asian and Caucasian women independently.

**Hypothesis** – Both the vitamin D$_2$ and vitamin D$_3$ interventions would lead to a change in blood lipid concentrations associated with a decreased risk of CVD, in both ethnic groups, with the vitamin D$_3$ intervention groups showing a greater change.
5.2 METHODS

5.2.1 Participants and Study Protocol

Participants were recruited as part of the D2-D3 Study and followed the study protocol as described in Chapter 2.1. In summary, healthy South Asian and Caucasian women aged 20-65 years were randomised to receive placebo, 15 µg/d vitamin D$_2$ or 15µ g/d vitamin D$_3$, delivered via fortified foods, for a 12-week period and attended three study visits for data collection; week 0, week 6 and week 12.

5.2.2 Relevant Data

The data relevant to CVD and therefore included in the analysis include age, BMI, body fat percentage, waist:hip ratio and blood pressure. The analysis also included the serum 25OHD concentrations (and metabolites) and plasma lipid concentrations that were taken at all three study visits.

5.2.3 Statistical Analysis

All statistical analyses were performed with the use of SPSS® software (version 22.0; SPSS Inc., Chicago, IL). Differences were considered to be significant at $p<0.05$. Data were checked for normality using the Kolmogorov-Smirnov test if sample size was $>50$ or Shapiro-Wilkinson otherwise.

Comparisons between ethnic groups at baseline were conducted using T-Tests where data were normally distributed, and Mann-Whitney U tests were data were not normally distributed. Partial correlations, controlling for relevant variables, were used to assess relationship between 25OHD concentrations and variables.

To compare lipid profiles at week 0, week 6 and week 12 within each treatment group, in South Asian and Caucasian cohorts independently, one-way repeated-measures ANOVA were conducted where data were normally distributed, and Friedman Tests followed by post-hoc Wilcoxon Signed Rank Tests were used where data were not normally distributed.
5.3 RESULTS

5.3.1 Baseline Anthropometry and Biochemistry

A total of 90 South Asian and 245 Caucasian women were recruited as part of the D2-D3 Study, and included in these analyses.

The South Asian cohort were significantly younger, had a higher BMI, higher body fat percentage and higher waist:hip ratio (WHR) than the Caucasian cohort. However, the South Asian cohort also had a significantly lower systolic blood pressure and 25OHD concentrations than the Caucasian cohort. Total cholesterol, HDL cholesterol and LDL cholesterol were all significantly lower in the South Asian cohort than the Caucasian cohort (Table 5.1).

| Table 5.1 Anthropometric values and biochemistry measurements, within and between South Asian and Caucasian women |
|-----------|--------|--------|--------|--------|-----------|
|          | South Asian | Caucasian | T-Test/M-Whitney |
|          | n | Mean | SD | n | Mean | SD | p-value |
| Age (years) | 90 | 37.07 | 10.32 | 245 | 45.71 | 12.24 | <0.001b |
| BMI (kg/m²) | 90 | 25.31 | 4.37 | 245 | 23.57 | 3.46 | <0.001b |
| Bodyfat (%) | 90 | 32.64 | 6.96 | 243 | 28.97 | 6.31 | <0.001b |
| Waist:Hip Ratio | 90 | 0.84 | 0.08 | 245 | 0.79 | 0.07 | <0.001b |
| Systolic BP (mmHg) | 89 | 112.96 | 13.31 | 245 | 120.35 | 14.85 | <0.001b |
| Diastolic BP (mmHg) | 89 | 76.43 | 9.81 | 245 | 78.85 | 9.69 | 0.045b |
| Total 25OHD (nmol/L) | 90 | 27.55 | 22.25 | 245 | 60.28 | 26.54 | <0.001b |
| Triglyceride (mmol/L) | 90 | 1.14 | 0.77 | 244 | 1.02 | 0.41 | 0.349b |
| Total Cholesterol (mmol/L) | 90 | 4.96 | 0.89 | 244 | 5.46 | 1.02 | <0.001a |
| HDL (mmol/L) | 90 | 1.49 | 0.29 | 244 | 1.85 | 0.40 | <0.001b |
| LDL (mmol/L) | 90 | 2.95 | 0.73 | 244 | 3.16 | 0.89 | 0.031b |
| NEFA (mmol/L) | 90 | 0.52 | 0.21 | 244 | 0.51 | 0.23 | 0.636b |

Key: BMI = body mass index, BP = blood pressure, HDL = high density lipoprotein cholesterol, LDL = low density lipoprotein cholesterol, NEFA = non-esterified fatty acids

a normally distributed analysed by t-test, b notnormally distributed analysed by Mann-Whitney U

5.3.2 Association between 25OHD and Lipid Profile

There were no significant associations between 25OHD concentrations and any of the plasma lipid and lipoprotein concentrations in both the South Asian and Caucasian cohorts, although there was a trend towards a positive association between 25OHD and triglyceride concentrations in the
Caucasian women, and a trend towards a positive association between 25OHD and HDL cholesterol concentrations in the South Asian women (Table 5.2).

Table 5.2 Partial correlation, controlling for age, BMI and body fat percentage, between 25OHD and lipid concentrations

<table>
<thead>
<tr>
<th></th>
<th>South Asian n85</th>
<th>Caucasian n236</th>
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<tbody>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>Correlation</td>
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</tr>
<tr>
<td></td>
<td>p-value</td>
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</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>Correlation</td>
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<tr>
<td></td>
<td>p-value</td>
<td>0.664</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>Correlation</td>
<td>0.201</td>
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<tr>
<td></td>
<td>p-value</td>
<td>0.062</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>Correlation</td>
<td>-0.01</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.927</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>Correlation</td>
<td>0.086</td>
</tr>
<tr>
<td></td>
<td>p-value</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Key: HDL = high density lipoprotein cholesterol, LDL = low density lipoprotein cholesterol, NEFA = non-esterified fatty acids

5.3.3 Changes in Anthropometrics and Lipid Profile

5.3.3.1 At Baseline (Week 0)

There were no significant differences in any of the anthropometric measurements or blood lipid concentrations between intervention groups. The only significant difference between intervention groups, within each ethnic group independently, was in NEFA concentrations in the South Asian cohort with NEFA concentrations in the vitamin D<sub>2</sub> group significantly lower than the vitamin D<sub>3</sub> group (p=0.007) (Table 5.3).
Table 5.3 Anthropometric, serum 25OHD and plasma lipid concentrations at all three study visits and changes over time, with ethnic groups split

<table>
<thead>
<tr>
<th>Measure</th>
<th>Ethnicity</th>
<th>Week 0</th>
<th>Week 6</th>
<th>Week 12</th>
<th>Change Over Time&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>South Asian</td>
<td>Caucasian</td>
<td>South Asian</td>
<td>Caucasian</td>
<td>South Asian</td>
</tr>
<tr>
<td>Triglyceride (mmol/L)</td>
<td>Placebo</td>
<td>N: 17 M: 1.33 M [1.49]</td>
<td>N: 15 M: 1.30 M [1.08]</td>
<td>N: 14 M: 1.05 M [0.37]</td>
<td>N: 45 M: 1.10 M [0.56]</td>
</tr>
<tr>
<td></td>
<td>Vit D2</td>
<td>N: 35 M: 1.1 M [0.46]</td>
<td>N: 30 M: 1.08 M [0.41]</td>
<td>N: 27 M: 1.08 M [0.44]</td>
<td>N: 92 M: 1.09 M [0.34]</td>
</tr>
<tr>
<td></td>
<td>Vit D3</td>
<td>N: 38 M: 1.09 M [0.48]</td>
<td>N: 24 M: 1.35 M [0.71]</td>
<td>N: 22 M: 1.35 M [0.68]</td>
<td>N: 91 M: 1.19 M [0.64]</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>Placebo</td>
<td>N: 17 M: 4.85 M [0.74]</td>
<td>N: 15 M: 4.68 M [0.48]</td>
<td>N: 14 M: 4.92 M [0.55]</td>
<td>N: 45 M: 5.41 M [1.06]</td>
</tr>
<tr>
<td></td>
<td>Vit D3</td>
<td>N: 38 M: 5.01 M [0.92]</td>
<td>N: 24 M: 5.31 M [0.92]</td>
<td>N: 22 M: 5.25 M [0.87]</td>
<td>N: 91 M: 5.58 M [1.13]</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>Placebo</td>
<td>N: 17 M: 1.46 M [0.34]</td>
<td>N: 15 M: 1.39 M [0.29]</td>
<td>N: 14 M: 1.51 M [0.30]</td>
<td>N: 45 M: 1.83 M [0.30]</td>
</tr>
<tr>
<td></td>
<td>Vit D2</td>
<td>N: 35 M: 1.47 M [0.30]</td>
<td>N: 30 M: 1.44 M [0.29]</td>
<td>N: 27 M: 1.49 M [0.39]</td>
<td>N: 92 M: 1.80 M [0.40]</td>
</tr>
<tr>
<td></td>
<td>Vit D3</td>
<td>N: 38 M: 1.52 M [0.25]</td>
<td>N: 24 M: 1.51 M [0.33]</td>
<td>N: 22 M: 1.54 M [0.31]</td>
<td>N: 91 M: 1.81 M [0.41]</td>
</tr>
<tr>
<td>LDL (mmol/L)</td>
<td>Placebo</td>
<td>N: 17 M: 2.77 M [0.54]</td>
<td>N: 15 M: 2.69 M [0.53]</td>
<td>N: 14 M: 2.93 M [0.55]</td>
<td>N: 45 M: 3.09 M [0.96]</td>
</tr>
<tr>
<td></td>
<td>Vit D2</td>
<td>N: 35 M: 3.00 M [0.74]</td>
<td>N: 30 M: 2.93 M [0.72]</td>
<td>N: 27 M: 2.92 M [0.89]</td>
<td>N: 92 M: 3.21 M [0.86]</td>
</tr>
<tr>
<td></td>
<td>Vit D3</td>
<td>N: 38 M: 2.99 M [0.79]</td>
<td>N: 24 M: 3.19 M [0.75]</td>
<td>N: 22 M: 3.10 M [0.84]</td>
<td>N: 91 M: 3.23 M [0.98]</td>
</tr>
<tr>
<td>NEFA (mmol/L)</td>
<td>Placebo</td>
<td>N: 17 M: 0.52 M [0.16]</td>
<td>N: 15 M: 0.40 M [0.20]</td>
<td>N: 14 M: 0.46 M [0.21]</td>
<td>N: 45 M: 0.51 M [0.29]</td>
</tr>
<tr>
<td></td>
<td>Vit D2</td>
<td>N: 35 M: 0.44 M [0.19]</td>
<td>N: 30 M: 0.46 M [0.19]</td>
<td>N: 27 M: 0.43 M [0.24]</td>
<td>N: 92 M: 0.50 M [0.24]</td>
</tr>
<tr>
<td></td>
<td>Vit D3</td>
<td>N: 38 M: 0.59 M [0.23]</td>
<td>N: 24 M: 0.54 M [0.20]</td>
<td>N: 22 M: 0.44 M [0.20]</td>
<td>N: 91 M: 0.54 M [0.30]</td>
</tr>
</tbody>
</table>

Key: M = mean, SD = standard deviation, <sup>a</sup> normally distributed analysed by repeated-measures ANOVA, <sup>b</sup> not-normally distributed analysed by Friedman Test with post-hoc Wilcoxon Signed Rank Test (adj p-value = 0.017), <sup>1</sup> significantly different from Week 0, <sup>2</sup> significantly different from week 6, <sup>3</sup> significantly different from week 12. Red indicates significance.
5.3.3.2 Changes in Anthropometrics

As shown in Table 5.3, there was a significant effect of intervention over time on BMI in both the Caucasian vitamin D2 and vitamin D3 groups, with the vitamin D2 group showing a significantly higher BMI at week 6 and week 12 compared to baseline. Although the vitamin D3 group demonstrated a significant effect of intervention over time, post-hoc analyses showed no significant differences between intervention groups at the adjusted significance levels of p<0.017. There was also a significant effect of intervention over time on body fat percentage in both the South Asian and Caucasian vitamin D3 groups, with the Caucasian group showing a significantly lower BMI at week 6 compared to baseline. Again, within the South Asian cohort there were no significant differences between visits in post-hoc analyses.

5.3.3.3 Changes in 25OHD Concentrations

As expected, and presented in Chapter 3.3.8, there was a significant effect of intervention over time on serum 25OHD concentrations in all groups, across both ethnic groups (Figure 3-3).

Both the South Asian and Caucasian placebo groups saw a significant decrease in 25OHD over time, with 25OHD concentrations at week 6 and week 12 significantly lower than at baseline for both ethnic groups (p<0.001 in all cases) and week 12 significantly higher than week 6 within the Caucasian (p<0.001). In contrast, the vitamin D2 and D3 intervention in both ethnic groups showed a significant increase in 25OHD over time, with both ethnic groups on the vitamin D2 and vitamin D3 intervention showing significantly higher 25OHD concentrations at week 6 and 12 compared with baseline (p<0.001 in all cases), and the vitamin D3 intervention for both ethnic groups also showed significantly higher 25OHD concentrations at week 12 compared to week 6 (SA p=0.033, Cauc p<0.001).
5.3.3.4 Changes in Lipid Profile

5.3.3.4.1 Triglyceride Concentrations

There was a significant effect of time on triglyceride concentrations in both the vitamin D$_2$ and vitamin D$_3$ intervention groups among the Caucasian cohort, with triglyceride concentrations at week 6 and week 12 significantly higher than at baseline. There was no effect of intervention over time on triglyceride concentrations within the South Asian cohort (Table 5.3 and Figure 5-1).

Figure 5-1 Triglyceride concentrations [mean ±SEM] at week 0, 6 and 12, within intervention groups split by ethnicity

$^*$ indicates significant difference compared to week 0 (p<0.05)
5.3.3.4.2 Total Cholesterol Concentrations

There was a significant effect of intervention over time on total cholesterol concentrations within the South Asian placebo intervention group, with week 12 total cholesterol significantly higher than week 6. There was also a significant effect of intervention over time on total cholesterol within the Caucasian vitamin D\textsubscript{2} and vitamin D\textsubscript{3} group, in both cases total cholesterol at week 6 was significantly higher than at baseline (Table 5.3, Figure 5-2).

![Total Cholesterol Concentrations](image)

**Figure 5-2 Total cholesterol concentrations [mean ±SEM] at week 0, 6 and 12, within intervention group split by ethnicity**

\(^0\) indicates significant difference compared to week 0, \(^6\) indicates significant difference compared to week 6 (\(p<0.05\))
5.3.3.4.3 NEFA Concentrations

There was a significant effect of intervention over time on NEFA concentrations in the South Asian vitamin D3 group, with NEFA concentrations at week 12 significantly lower than at baseline (Table 5.3, Figure 5-3).

![NEFA Concentrations](image)

Figure 5-3 NEFA concentrations [mean ±SEM] at week 0, 6 and 12, within intervention group split by ethnicity

° indicates significant difference compared to week 0 (p<0.05)

5.3.3.4.4 HDL and LDL Cholesterol Concentrations

There was also a significant effect of intervention over time on HDL cholesterol concentrations in the South Asian vitamin D3 group, and a significant effect on LDL cholesterol concentrations in the Caucasian vitamin D2 group, however in post-hoc analyses there were no significant differences between visits (Table 5.3).
5.4 DISCUSSION

5.4.1 Markers of CVD Risk between Ethnic Groups

Studies of migrant South Asian populations have shown an increased risk of cardiovascular death among South Asians compared with other ethnic groups (McKeigue et al. 1989; Harding et al. 2003; McCarty 2009). In this current study, the South Asian women were of a shorter height, higher BMI, higher body fat percentage and higher waist:hip ratio than Caucasian women. This would suggest that the South Asian women have a higher risk of CVD than the Caucasian women, especially as for a given BMI South Asians have greater adiposity than Caucasians. At a BMI >21 kg/m$^2$ there is an association with dyslipidemia in the South Asians, whereas comparable changes do not occur in the Caucasians until their BMI is >30 kg/m$^2$ (Razak et al. 2007). Furthermore, a consultation that reviewed associations between BMI and body fat percentage with CVD risk found that the proportion of Asian people with CVD who had a healthy BMI (<25 kg/m$^2$) was substantial (WHO 2004). Interestingly, the South Asian women did nevertheless have lower blood pressure, lower total cholesterol and lower LDL cholesterol than the Caucasian cohort, which was not expected, and this may be due to the younger age of the South Asian cohort (Franklin 1999).

5.4.2 Associations between 25OHD and Markers of CVD Risk between Ethnic Groups

This study showed no associations between 25OHD and lipid concentrations within either ethnic group, which is inconsistent with findings of other studies that have shown associations between 25OHD and risk of CVD, but similar to findings from meta-analyses that have shown less consistent effects of vitamin D supplementation on lipid concentrations, as were discussed in Chapter 5.1.3. Some previous studies have shown no association between 25OHD concentrations and total cholesterol or HDL cholesterol concentrations (Forouhi et al. 2008), which concurs with our findings, yet other studies have shown positive correlations between 25OHD and HDL cholesterol concentrations (Auwerx et al. 1992). Additionally, 25OHD concentrations were inversely association with triglyceride concentrations in 6,810 Caucasians, in the 1958 Birth Cohort Study (Hyppönen et al. 2008), which contradicts our findings. In South Asian cohorts specifically, no associations between 25OHD concentrations and triglyceride, total cholesterol, HDL cholesterol and LDL cholesterol concentrations were found in Bangladeshi men and women, when controlling for risk factors (John et al. 2005), which is consistent with our findings in the South Asian women.
5.4.3 Effect of Vitamin D Supplementation on Markers of CVD Risk

There were no consistent changes in lipid concentrations in response to the intervention. The only significant beneficial change to blood lipid was an increase in HDL cholesterol in the South Asian cohort in response to vitamin D₃. However, despite it being statistically significant, it was not clinically significant as the mean increase was only 0.02 mmol/L. The South Asians in the placebo intervention showed a significant increase in total cholesterol. These two findings suggest that change in 25OHD concentrations has an effect on CVD risk, as they showed a negative effect on total cholesterol with decreasing 25OHD concentrations, and a positive effect on HDL cholesterol with increasing 25OHD concentrations, with the South Asian vitamin D₃ intervention group showing the greatest increase in 25OHD concentrations out of the six intervention groups. Caucasians in both the vitamin D₂ and D₃ groups showed significant increases in triglyceride and total cholesterol concentrations, with the vitamin D₂ group also showing increases in LDL cholesterol concentrations. This significant increase on LDL cholesterol has also been shown previously in a meta-analyses of 12 clinical trials that examined the effects of vitamin D supplementation on lipid profiles (Wang et al. 2012).

An important thing to note in the interpretation of these results, is that although some findings were statistically significant, the clinical relevance of changes shown was not significant. Across all three study visits the mean triglyceride and HDL cholesterol concentrations of all intervention groups in both ethnicities remained below or above the cut-offs for CVD risk, at >2 mmol/L and <1 mmol/L respectively. For both total cholesterol and LDL-cholesterol the mean concentrations across the visits were only above or below the cut-offs for CVD risk, respectively, if they had been so at baseline and therefore clinical risk status was unaffected by the intervention. Nevertheless, the findings of an increase triglyceride and LDL-cholesterol concentrations do warrant further research, particularly in population groups at greater risk of CVD, where even slight increases may be detrimental.

5.4.4 Strengths and Limitations

This study was completed during the winter-spring period to control for the impact of sunlight exposure and seasonality on 25OHD and lipid concentrations. However, a limitation of this study was the population sample. Both the Caucasian and South Asian cohorts were of relatively good health, and participants were only recruited if they had a BMI <30 kg/m². Therefore plasma lipid concentrations were reasonably healthy across all participants, giving less opportunity for an intervention to improve CVD risk.
5.4.5 Concluding Remarks and Areas for Further Research

Even though there is extensive evidence of associations between 25OHD and lipid concentrations in observational studies, this study was not able to reproduce some of the findings that have been shown in large Caucasian populations. A possible explanation for this could be the health of our cohort and the narrow ranges of 25OHD concentrations between participants at baseline. This study, along with other supplementation trials (Wang et al. 2012), has not shown a clear causal effect between 25OHD and blood lipid concentrations. It is possible that our study cohort were too healthy and the dose of vitamin D$_2$ and/or D$_3$ was too low to show any benefits.

To conclude, this study has shown that there is no clinical benefit of 12-weeks supplementation with either vitamin D$_2$ or vitamin D$_3$ on lipid concentrations in South Asian and Caucasian female adult populations, who are in good health and at low risk of CVD at baseline. Further work to examine associations between baseline CVD markers and response to supplementation, adjusted for quartiles of BMI would be a useful area for future secondary analyses.
6.1 INTRODUCTION

6.1.1 Gene Expression and Regulation

Gene expression is the mechanism by which the genetic code, the nucleotide sequence, from a gene is used to synthesize a functional gene product, a protein, which go on to perform essential functions as enzymes, hormones or receptors. This process involves two key stages; transcription and translation. Some genes are expressed continuously, as they produce proteins involved in basic metabolic functions, but the expression of many genes can be regulated to increase, decrease or inhibit the production of the functional gene product and influence biological processes. Measuring gene expression can help to determine disease risk or response to treatment.

6.1.2 Mechanisms of Vitamin D Regulating Gene Expression

The role of vitamin D in regulating gene expression has become increasingly evident since the discovery of the transcription factor vitamin D receptor (VDR) (Brumbaugh and Haussler, 1974; Brumbaugh and Haussler, 1974). Vitamin D exerts its biological effects in target tissues via the action of its active form, 1,25-dihydroxyvitamin D (1,25(OH)₂D), which acts as a ligand to the VDR (Carlberg and Molnar, 2012). When the VDR is activated by its ligand 1,25(OH)₂D, it acts as a transcription factor (Macdonald et al. 1994). The regulation of genes by ligand-bound VDR can be modulated positively or negatively.

The classic, physiological role of 1,25(OH)₂D after binding to the VDR is the regulation of calcium and phosphate homeostasis and bone mineralisation (DeLuca 2004), and enterocytes, osteoblasts, parathyroid and distal renal tubule cells that are involved in calcium and phosphate metabolism express the VDR (Jones 1998). VDRs have also been shown to be expressed in leucocytes (Soldati et al. 2004) macrophages, lymphocytes, skin keratinocytes, pancreatic ß-islet cells, ovarian tissue, mammary epithelium, neuronal tissue, lung, gonads, prostate, placenta, and adipose tissue (Jones 1998; Norman 2008). This discovery of the VDR in many other cells and tissues that are not considered targets of vitamin D action has led to the recognition that the VDR, and therefore vitamin D, has a role to play in many more processes than previously thought. There are estimations that VDR activation may regulate the expression of 0.5-5% of the total human genome (100-1250 genes) (Hossein-Nezhad et al. 2013; Holick 2008; Zhang et al. 2011; Yu & Cantorna 2011), including several genes that encode cytochrome P450 enzymes that are essential in vitamin D metabolism (Drocourt et al. 2002). The evidence so far has shown that the non-calcaemic actions of the VDR-ligand include cell proliferation, differentiation and immune-modulatory functions (Ingraham et al. 2008; Verstuyf et al. 2010).
6.1.3 Treatment/Supplementation with Vitamin D and Gene Expression

6.1.3.1 In vitro Analyses

Transcriptome-wide analyses has shown that the mRNA expression of >1,000 genes in cell cultures are changed after stimulation with 1,25(OH)\textsubscript{2}D\textsubscript{3}, although findings vary by cell type (Ramagopalan et al. 2010; Heikkinen et al. 2011; Kovalenko et al. 2010; Nurminen et al. 2015).

In a genome-wide microarray analysis of human osteoblasts the expression of 229 genes were altered following treatment with 1,25(OH)\textsubscript{2}D\textsubscript{3}, with 226 genes significantly up-regulated and 3 genes significantly down-regulated (Ramagopalan et al. 2010).

6.1.3.2 In vivo Analyses

There have been a couple of vitamin D supplementation trials that have examined the expression of selected genes before and after the intervention. Carlsberg and colleagues (2013) studied the expression of primary vitamin D target genes in peripheral blood mononuclear cells (PBMCs) collected at the start and end of a 5-month vitamin D\textsubscript{3} supplementation trial in 71 elderly (>60 yrs) pre-diabetic individuals in Finland. Participants were randomised to either placebo (n=22), 40μg vitamin D\textsubscript{3} (n=25) or 80μg vitamin D\textsubscript{3} (n=24) daily for the 5 months. They found no association between the changes in 25OHD\textsubscript{3} concentrations and the changes in mRNA expression of CD14 or THBD in analyses of all 71 participants. However, when the 35 participants with the greatest change in 25OHD\textsubscript{3} were used in subset analyses, there was a significant positive correlation between change in 25OHD\textsubscript{3} and expression of both CD14 and thrombomodulin (THBD) genes. The authors suggested that CD14 and THDR could therefore be used as biomarkers to assess the transcriptomic response to vitamin D\textsubscript{3} supplementation.

In further analyses of this dataset, 12 VDR target genes (whose expression in PBMCs at the start and end of the study correlated with the changes in 25OHD\textsubscript{3} concentrations) were analysed to see if their expression could determine responsiveness to vitamin D\textsubscript{3} supplementation (Saksa et al. 2015). They observed no significant correlation between the 12 VDR target genes change in expression and change in 25OHD\textsubscript{3}, when analysing data from all 71 participants. However, following a stepwise elimination of up to 50% of the participants, highly significant associations were shown for all 12 VDR target genes (Saksa et al. 2015). A further 12 VDR target genes, whose expression was also significantly associated with changes in 25OHD\textsubscript{3}, were also identified from this cohort in further analyses (Vukic et al. 2015).

Jorde and colleagues examined the effect of supplementation with placebo (n=37) or 500-1,000 μg vitamin D\textsubscript{3} per week (n=40) for 12 months, on the expression of 12 non-coding RNAs (microRNAs/miRNA), and were unable to demonstrate any consistent significant change in expression.
in response to the vitamin D$_3$ (Jorde et al. 2012). However, there study was limited by the number of miRNAs included in analyses and the low expression of miRNAs in plasma.

The first and only published study to have studied the effect of vitamin D supplementation on genome-wide gene expression was completed by Hossein-Nezhad and colleagues (2013). They conducted a small, randomised-controlled pilot study in eight participants; three who received 10 μg (400 IU) and five who received 50 μg (2,000 IU) of vitamin D$_3$ daily for two months. At baseline they identified 66 genes that were differentially expressed between those with 25OHD concentrations <50 nmol/L (n4) and >50 nmol/L (n4). Of these 66 genes, there was at least a 1.5 fold increase in gene expression of 14 genes and at least a 1.5 fold decrease in 52 genes in those with 25OHD concentrations <50 nmol/L (mean 40.5 nmol/L) compared to those with 25OHD concentrations >50 nmol/L (mean 68.75 nmol/L). After supplementation, which resulted in mean 25OHD concentrations of 62.75 nmol/L and 88 nmol/L in the groups, respectively, there was no longer a significant difference between the groups in the expression of those 66 genes. To explore the impact of vitamin D$_3$ supplementation on gene expression, both the 10 μg and 50 μg groups were combined, as the researchers found no dose-dependent difference in the alteration in gene expression between the groups. They identified 291 genes that had a 1.5 fold alteration in expression from baseline to follow-up in response to vitamin D$_3$ supplementation. Of these 291 genes, 82 were down-regulated and 209 were up-regulated. Forty-seven of the 291 genes had not previously been reported and were discovered in these analyses. They identified that the identified genes affected the following pathways; transcription regulation, immune function, cell cycle activity, epigenetic modification, DNA regulation, DNA repair and cellular response to stress.

6.1.4 Justification of Study

As we have seen in Chapter 3.3.8, total change in 25OHD differs between those who were given vitamin D$_2$ compared to those given vitamin D$_3$, and between the Caucasian and South Asian populations, and there is also inter-individual variability within ethnic groups. This study aimed to investigate the effects of vitamin D$_2$ and/or vitamin D$_3$ supplementation, through fortification, on gene expression in order to associate specific signal transduction and metabolic pathways to respective vitamin D responses, and to investigate whether both forms of vitamin D activate the same biological response, as this has not been studied previously.
Identifying which genes are up- or down-regulated in response to vitamin D$_2$ and D$_3$ supplementation will not only potentially suggest mechanisms for the superior efficacy of vitamin D$_3$ compared to vitamin D$_2$, but also potentially identify new pathways that have not previously been found to be affected or to respond to vitamin D supplementation.

### 6.1.5 Aims and Hypothesis

**Aim 12** - To determine the effect of 15 µg/d (600 IU/d) vitamin D$_2$ vs. vitamin D$_3$ on gene expression, through leucocyte transcriptome-wide analysis, in both South Asian and Caucasian women.

**Hypothesis** – Both the vitamin D$_2$ and vitamin D$_3$ interventions would lead to the same changes in gene expression over the study period, but the differences over time would be greater in those receiving vitamin D$_3$ compared to those receiving vitamin D$_2$ in both ethnic groups.

### 6.2 METHODS

#### 6.2.1 Participants

Individuals were recruited as part of the D2-D3 Study, which is described in detail in Chapter 2. In summary, the D2-D3 Study recruited both South Asian and Caucasian women and randomised them to one of three intervention groups for 12-weeks; placebo, 15 µg/d vitamin D$_2$ or 15µg/d vitamin D$_3$.

Of the D2-D3 Study participants who provided separate consent to the D2-D3 Genetic Study, a sub-set of 50 participants were selected for microarray analyses. Of these 50 participants, 28 were to be Caucasian and 22 were to be South Asian, with an equal representation from the vitamin D$_2$ and vitamin D$_3$ intervention within each ethnic group. Selection within each ethnic group and intervention group was based on percentage change in 25OHD status in response to supplementation, and both ‘high responders’ (>50% increase in 25OHD concentrations) and ‘low responders’ (<50% increase in 25OHD concentrations) were selected from both vitamin D$_2$ and D$_3$ supplementation groups to allow for comparisons in future analyses. The rationale for this was that comparison of the highest and lowest responding groups would maximize the chance of observing differences in gene expression among subjects.
6.2.2 Study Protocol

The D2-D3 Study protocol is described in detail in Chapter 2.1. In addition to the measurement of anthropometrics and the collection of a fasted blood sample for the measurement of serum 25OHD concentrations and vitamin D metabolites, those who consented to the D2-D3 Genetic Study also had an additional 2.5ml blood sample collected in a PAXgene Blood RNA tube (Becton Dickinson) at all three study visits. PAXgene Blood RNA Tubes were stored at 15-25°C for 24 hours, followed by a -20°C freezer for 24 hours and then into a -80°C freezer for long-term storage. Prior to RNA extraction, samples were defrosted at room temperature for 2 hours.

6.2.3 RNA Extraction, Labelling, and Microarray Hybridization and Processing

Whole peripheral blood (2.5ml) was collected using PAXgene Blood RNA tubes (Becton Dickinson) at selected time points. Total RNA was isolated using PAXgene Blood RNA Kit (QIAGEN, PreAnalytiX cat 762164) and a QiaCube robot (Qiagen) or manually, following the manufacturers protocol. cRNA was synthesized and fluorescently labeled with Cy3-CTP from 200ng of total RNA using the Low Input Quick Amp Labeling Kit, One Color (Agilent). Labelled cRNA (1.6 ug) was hybridised on a Sure Print G3 Human Gene Expression 8x60K v2 microarray slide (Agilent). Standard manufacturer’s instructions for one-colour gene-expression analysis were followed for labeling, hybridisation and washing steps.

Extracted RNA was quantified and the A260/280 nm and A260/230 nm ratios were determined using NanoDrop ND2000 spectrophotometer (ThermoScientific). RNA quality was assessed using the Bioanalyser 2100 (Agilent Technologies). Only RNA samples with an RNA Integrity Number (RIN) >7 were subjected to microarray analysis; the majority of the samples had a RIN score of >8.

To minimize undesirable differences in gene expression derived from batch effects, the samples from 8 participants (each with three samples) were processed on three 8 x 60K Human Gene Expression microarrays in a single experiment. Microarrays were hybridized at 65°C for 17 hours in an Agilent hybridization oven with rotisserie at 10 rpm. The microarrays were washed with Agilent Wash Buffer 1, prewarmed Wash Buffer 2 (37°C), according to the manufacturer’s instruction. The processed microarrays were scanned using an Agilent Microarray Scanner with a resolution of 2 μm. The images derived from each slide were scanned and imported into the Agilent Feature Extraction Software (version 11.5.1.1) for image analysis and data extraction.
6.2.4 Data Handling and Statistical Analyses

Feature extraction files were read into R, the gene expression matrix (GEM) was defined as log₂ of the “gProcessedSignal”.

The annotation file corresponding to Agilent human 8x66K (downloaded from Agilent eArray tool) was used to annotate each row of the GEM. [This was conducted by Dr C. Möller-Levet, Faculty Bioinformatics Officer]

6.2.4.1 Pre-processing

1. Quantile normalisation was performed using “normalizeBetweenArrays” function in R Bioconductor “limma” package (Smyth and Speed, 2003).
2. Control probes were filter out.
3. Non-control replicated probe were averaged along with their flags information.
4. Filtering of flagged probes was done specifically for each analysis/comparison performed. Probes flagged by Agilent Feature Extraction (version 11.5.1.1) in more than 2/3 of the total number of samples used in a specific comparison were filtered out.

6.2.4.2 Comparison tests

An ANOVA between and within groups were performed. This was implemented as a repeated measures mixed model using the “anova” function (car R package, (Fox and Weisberg, 2011) on the “lmer” model (lme4 R package, (Bates et al. 2015) where ethnicity, treatment, visit and score are fixed main effects, the simple interactions of ethnicity, treatment and visit are considered and subjects are random effects. Expression profiles across the three visits of significant sets of probes were clustered using K-means (Hartigan et al. 1979).

In all cases p-values were corrected for multiplicity using the Benjamini and Hochberg (BH) method via the “p.adjust” function in R (Benjamini and Hochberg, 1995).

6.2.4.3 Pathway Analysis

To determine the biological functions represented by the changes in gene expression, lists of genes and fold-change in expression were exported into MetaCore™ (GeneGo, Thomson Reuters). Enrichment analysis was performed in MetaCore™ using each exported list to determine/compare top functional processes.
6.3 RESULTS

6.3.1 Participant Characteristics

Microarrays were successfully completed for 50 participants; \( n = 25 \) from the vitamin D\(_2\) intervention and \( n = 25 \) from the vitamin D\(_3\) intervention. At baseline, there were no significant differences in anthropometric measurements, or total 25OHD, 25OHD\(_2\) and 25OHD\(_3\) concentrations at baseline between the vitamin D\(_2\) and D\(_3\) groups (Table 6.1). At visits 2 and 3, there were significant differences in total 25OHD, 25OHD\(_2\) and 25OHD\(_3\) concentrations between the vitamin D\(_2\) and vitamin D\(_3\) groups (Table 6.1 and Figure 6-1).

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Vitamin D(_2)</th>
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<th>T-Test p-value</th>
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<td>25</td>
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<td>Age (yrs)</td>
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<td>Height (m)</td>
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<td>1.61 ±0.08</td>
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<tr>
<td>Weight (kg)</td>
<td>63.87 ±11.02</td>
<td>64.12 ±11.59</td>
<td>63.62 ±10.66</td>
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<tr>
<td>BMI (kg/m(^2))</td>
<td>24.13 ±4.51</td>
<td>24.74 ±4.92</td>
<td>23.52 ±4.07</td>
<td>ns</td>
</tr>
<tr>
<td><strong>Total 25OHD (nmol/L)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Week 0 (V1)</td>
<td>49.57 ±32.47</td>
<td>50.8 ±35.01</td>
<td>48.33 ±30.39</td>
<td>ns</td>
</tr>
<tr>
<td>Week 6 (V2)</td>
<td>71.51 ±24.17</td>
<td>62.78 ±20.79</td>
<td>80.24 ±24.54</td>
<td>0.009</td>
</tr>
<tr>
<td>Week 12 (V3)</td>
<td>75.80 ±25.64</td>
<td>64.53 ±20.07</td>
<td>87.06 ±25.97</td>
<td>0.001</td>
</tr>
<tr>
<td>Total Change: V1 to V3</td>
<td>26.24 ±31.92</td>
<td>13.74 ±26.01</td>
<td>38.73 ±32.83</td>
<td>0.004</td>
</tr>
<tr>
<td>% Change: V1 to V3</td>
<td>124.0 ±154.2</td>
<td>83.6 ±113.9</td>
<td>164.3 ±179.3</td>
<td>0.063</td>
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<td><strong>25OHD(_2) (nmol/L)</strong></td>
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<td></td>
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<tr>
<td>Week 0 (V1)</td>
<td>1.57 ±1.28</td>
<td>1.49 ±1.33</td>
<td>1.65 ±1.25</td>
<td>ns</td>
</tr>
<tr>
<td>Week 6 (V2)</td>
<td>18.15 ±18.76</td>
<td>34.55 ±12.52</td>
<td>1.75 ±1.27</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Week 12 (V3)</td>
<td>21.89 ±23.68</td>
<td>42.02 ±17.28</td>
<td>1.76 ±1.19</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>25OHD(_3) (nmol/L)</strong></td>
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<td>Week 0 (V1)</td>
<td>4.79 ±32.53</td>
<td>49.31 ±34.86</td>
<td>46.68 ±30.68</td>
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<td>Week 6 (V2)</td>
<td>53.36 ±33.35</td>
<td>28.23 ±18.69</td>
<td>78.49 ±24.62</td>
<td>&lt;0.001</td>
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<tr>
<td>Week 12 (V3)</td>
<td>53.91 ±38.38</td>
<td>22.51 ±16.61</td>
<td>85.30 ±26.04</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Key: SD= standard deviation, BMI= body mass index, ns= non-significant
Figure 6-1 Quantities of 25OHD$_2$ and 25OHD$_3$ contributing to total 25OHD concentrations. 25OHD$_2$, 25OHD$_3$ and total 25OHD (sum of 25OHD$_2$ and 25OHD$_3$) concentrations (nmol/L) at V1 (week 0), V2 (week 6) and V3 (week 12) within all subjects (n50), the vitamin D$_2$ intervention subjects (n25), and the vitamin D$_3$ intervention subjects (n25).

6.3.1.1 Split by ethnicity

Of the 50 participants, 28 were Caucasian and 22 were South Asian. Participant characteristics and 25OHD concentrations of participants, split by ethnicity are shown in Table 6.2. Although cohorts split by ethnicity are not used in the analyses presented here, they will be in the future.

<table>
<thead>
<tr>
<th>Table 6.2 Participant characteristics and 25OHD concentrations (mean ±SD) of participants, split by ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
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</tr>
<tr>
<td>$n$</td>
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<tr>
<td>Age (yrs)</td>
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<td>BMI (kg/m$^2$)</td>
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<td>Week 0 (V1)</td>
</tr>
<tr>
<td>Week 6 (V2)</td>
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<tr>
<td>Week 12 (V3)</td>
</tr>
</tbody>
</table>

Key: SD= standard deviation, BMI= body mass index
6.3.2 Differences in Gene Expression across time: Vitamin D Intervention

6.3.2.1 Gene Expression

In analyses of all 50 participants combined (both vitamin D\textsubscript{2} and vitamin D\textsubscript{3} treated participants) at each study visit, there were significant differences in gene expression over time. A total of 1,129 probes, representing 1,045 genes, were significantly differentially expressed over the three study visits, with 880 of these probes being down-regulated across the visits, and 249 probes being up-regulated (Figure 6.2).

![Clustered heatmap demonstrating expression level of 1,129 probes at V1 (week 0), V2 (week 6) and V3 (week 12)](image)

Red indicates high level of expressions and blue indicates low levels of expression.

Probes with similar expression profiles over the three visits were clustered together, as shown in Figure 6-3. Clusters 1 and 3 (n880) were the probes that were down-regulated over the three visits/ 12 weeks, cluster 2 (n208) were up-regulated, and cluster 4 (n41) showed a slight down-regulation between weeks 0 and 6, followed by up-regulation between weeks 6 and 12.
6.3.2.2 Pathway Analysis

The most over-represented pathways within the down-regulated probes (Clusters 1 & 3), the up-regulated probes (Cluster 2), and the down-then-up-regulated probes (Cluster 4) clusters were identified through enrichment analysis using Metacore software for data mining and pathway analysis (Thomson Reuters).
6.3.2.2.1 Down-Regulated Probes/Genes – Cluster 1 & 3

Of the 880 probes that were down-regulated (cluster 1 & 3), the most over-represented pathways are shown in Figure 6-4. The most over-represented pathway is the ‘cell adhesion integrin inside-out signaling in neutrophils’, in which 22 of the 77 gene products involved in the pathway were differentially expressed (down-regulated) over time. This pathway is illustrated in Appendix 35 (see Quick Reference Guide, Appendix 36).

Figure 6-4 Top pathways identified in Cluster 1 and 3.
Histograms representing top pathways from the enrichment analysis of the gene lists identified in cluster 1 and 3. In each histogram, the longer the length of each bar the more significant (-log p-value) the pathway. The results are organised according to descending significance.
6.3.2.2 Up-Regulated Probes/Genes – Cluster 2

Of the 208 genes that were up-regulated, (cluster 2), the over-represented pathways are shown in Figure 6-5. The most over-represented pathway that is up-regulated is the ‘Development Slit-Robo signaling’, shown in Appendix 37.

![Figure 6-5 Top pathways identified in Cluster 2.](image)

Histograms representing top pathways from the enrichment analysis of the gene lists identified in cluster 2. In each histogram, the longer the length of each bar the more significant (-log p-value) the pathway. The results are organised according to descending significance.

6.3.2.2.3 Up-Regulated Probes/Genes – Cluster 4

Of the 41 genes that were initially down-regulated but overall up-regulated, (cluster 4), the over-represented pathways are shown in Figure 6-6. The most over-represented pathway is the ‘Cell adhesion - Role of CDK5 in cell adhesion’, shown in Appendix 38.

![Figure 6-6 Top pathways identified in Cluster 4.](image)

Histograms representing top pathways from the enrichment analysis of the gene lists identified in cluster 4. In each histogram, the longer the length of each bar the more significant (-log p-value) the pathway. The results are organised according to descending significance.
6.3.3 Differences in Gene Expression over Time: Vitamin D$_2$ Intervention

6.3.3.1 Gene Expression and Pathway Analysis

In the D2 treated participants ($n=25$) 115 probes/genes were differentially expressed between week 0 and week 12. Of these, 48 genes were up-regulated and 67 genes were down-regulated. When looking at the pathways of all 115 genes the over-represented pathways are shown in Figure 6-7. The most over-represented pathway was ‘Immune response IL-5 signaling’, followed by ‘Transcription Assembly of RNA polymerase II preinitiation complex on TATA-less promoters’, shown in Appendix 39 and Appendix 40, respectively.

![Figure 6-7: Top pathways identified following the vitamin D$_2$ intervention.](image)

Histograms representing top pathways from the enrichment analysis of the gene lists identified following vitamin D$_2$ intervention. In each histogram, the longer the length of each bar the more significant (-log p-value) the pathway. The results are organised according to descending significance.
6.3.4 Differences in Gene Expression over Time: Vitamin D\textsubscript{3} Intervention

6.3.4.1 Gene Expression and Pathway Analysis

In the D\textsubscript{3} treated participants (n = 25) 282 genes were differentially expressed between week 0 and week 12. Of these, 4 genes were up-regulated and 278 genes were down-regulated. When looking at the pathways of all 282 genes the over-represented pathways are shown in Figure 6-8. The most over-represented pathway was ‘Immune response PIP3 signaling in B lymphocytes’, followed by ‘Transcription Sin3 and NuRD in transcription regulation’, which are shown in Appendix 41 and Appendix 42, respectively.

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</tr>
</tbody>
</table>

Figure 6-8 Top pathways identified following the vitamin D\textsubscript{3} intervention. Histograms representing top pathways from the enrichment analysis of the gene lists identified following vitamin D\textsubscript{3} intervention. In each histogram, the longer the length of each bar the more significant (-log p-value) the pathway. The results are organised according to descending significance.

6.3.5 Similarities with previous studies

6.3.5.1 Hossein-Nezhad et al. 2013

A previous study in which participants were given 10 µg/d or 50 µg/d of vitamin D\textsubscript{3} for two months, identified 241 genes that were differentially expressed over time. Of those 241 genes, only seven genes overlapped with the 1,045 genes identified in response to vitamin D (vitamin D\textsubscript{2} and D\textsubscript{3} intervention combined) in the D2-D3 Study, as shown in Figure 6-9. The seven common genes identified were RBM12B, STK38, COPB2, HSPA4, IWS1, MFAP1 and RBM5.
6.3.5.2 VitDmet Study (Vukic et al. 2015; Saksa et al. 2015)

Following a 5-month intervention of placebo (n22), 40 μg/d vitamin D₃ (n25) or 80 μg/d vitamin D₃ (n24), the expression of 24 vitamin D receptor target genes has been associated with changes in 25OHD₃ concentrations (Vukic et al. 2015; Saksa et al. 2015). Of the 1,045 genes identified as differentially expressed following vitamin D (both vitamin D₂ and vitamin D₃ groups combined) in the D2-D3 Study, only 4 genes overlapped with those 24 genes identified in the two previous publications from the VitDmet study dataset (Vukic et al. 2015; Saksa et al. 2015), as shown in Figure 6-10. The four common genes identified were ITGAM, FBP1, CD14 and CD97.

Figure 6-10 Venn diagram showing overlap of genes identified in D2-D3 Study vs. Vukic et al. 2015 and Saksa et al. 2015.
Key: Carlberg genes: Vukic et al. 2015 and Saksa et al. 2015, CML ANOVA: D2-D3 Study.
6.3.5.3 Ramagopalan et al. (2010) in vitro study

Of the 229 genes identified as differentially expressed following treatment of human osteoblasts with 1,25(OH)₂D₃ in a previous study (Ramagopalan et al. 2010), only 16 genes overlapped with the 1,045 genes identified in response to vitamin D (vitamin D₂ and D₃ intervention combined) in the D2-D3 Study, as shown in Figure 6-11. The 16 genes that overlapped were PSAP, VDR, KLF13, ZC3H7A, SLC44A2, CD97, MKNK2, SULF2, RAC2, IQSEC1, GLB1, CAST, HIVEP2, HIPK2, ZHX2 and TRIB1.

![Venn diagram showing overlap of genes identified in D2-D3 Study vs Ramagopalan et al. 2010](image)

**Figure 6-11** Venn diagram showing overlap of genes identified in D2-D3 Study vs Ramagopalan et al. 2010
Key: Ramagopalan DE: Ramagopalan et al. 2010, CML ANOVA: D2-D3 Study

6.4 DISCUSSION

This genome-wide expression analysis provides the first insight into the changes in global transcriptomic activity that underlies the effects of vitamin D₂ supplementation and vitamin D₃ supplementation.

6.4.1 Vitamin D Intervention (D₂ and D₃ combined)

From the ANOVA analysis it was revealed that vitamin D supplementation caused a decreased expression of 880 genes and an increased expression of 249 genes, alongside an average increase in 25OHD concentrations from 49.6 ±32.5 nmol/L to 75.8 ±25.6 nmol/L. The pathways that were enriched, deduced from the numbers of differentially expressed genes from these pathways, in response to vitamin D supplementation included inflammatory processes, immune functions, transcriptional regulation and cytoskeletal remodeling. For example, the pathway ‘cell adhesion - integrin inside-out signaling in neutrophils’ (Appendix 35) which was down-regulated in response to vitamin D₃, is a key inflammatory process, and although inflammatory reactions can be beneficial and necessary for host defense, they need to be balanced and controlled to prevent harmful consequences.
and tissue destruction, including development of autoimmune diseases (Zarbock et al. 2012). The down-regulation of several gene products involved in this pathway, and therefore a down-regulation in leucocyte arrest, suggests an anti-inflammatory role for vitamin D. One of the up-regulated pathways was the ‘Development Slit-Robo signalling’ (Appendix 37) in which actin is up-regulated, and cytoskeletal remodeling is enhanced in response to vitamin D. These findings are consistent with previous smaller-scale *in vivo* studies (Hossein-Nezhad et al. 2013) that showed 25OHD₃ concentrations were associated with a change in expression of genes that were responsible for immune function, mineralization and bone development, transcription regulation and response to stress and DNA repair.

Unlike in this present D2-D3 Study dataset, previous studies, both *in vitro* and *in vivo*, have shown a greater number of genes up-regulated in response to vitamin D supplementation or 1,25(OH)₂D₃ stimulation. Hossein-Nezhad and colleagues (2013) showed that more genes were up-regulated (n209) in response to vitamin D₃ supplementation, than were down-regulated (n82). Previous in-vitro analyses have also shown a far greater number of genes to be up-regulated (n226) than down-regulated (n3) in response to stimulation of lymphoblastoid cell lines with 1,25(OH)₂D₃ for 36 hours (Ramagopalan et al. 2013). Vukic and colleagues (2015), on the other hand, showed that 10 of the 12 genes they examined were down-regulated, and so only two were up-regulated. However, they did not complete genome-wide analyses: they specifically selected 12 genes.

### 6.4.2 Independent effects of Vitamin D2 and Vitamin D3

The studies that have been published to date have only studied the effect of vitamin D₃ supplementation and 25OHD₃ concentrations, or the effect of stimulation of cells with 1,25(OH)₂D₃ *in vitro*, on gene expression. As shown in Chapter 3, vitamin D₃ is more effective than vitamin D₂ at raising total 25OHD concentrations, and therefore determining whether both forms of vitamin D have the same effects on gene expression is important in determining the biological effects of both forms and whether they do exert the same metabolic response.

In the vitamin D₂ intervention group 25OHD concentrations changed from 50.8 ±35.0 nmol/L to 64.5 ±20.1 nmol/L over the 12-weeks, and in that time 115 probes were identified as differentially expressed at week 12 compared to baseline. Of those 115, 48 were up-regulated and 67 were down-regulated. Whereas, in the vitamin D₃ intervention group 25OHD concentrations changed from 48.3 ±30.4 nmol/L to 87.1 ±26.0 nmol/L over the 12-weeks, and 282 probes were shown to be
differentially expressed at week 12 compared with baseline. Of those 282, only 4 were up-regulated but 278 probes were down-regulated.

This is the first study of its kind, to have examined gene expression in response to vitamin D\textsubscript{2} supplementation compared with vitamin D\textsubscript{3} supplementation, and so there are no previous studies with which to compare the results. However, the findings within the vitamin D\textsubscript{3} intervention corroborate previous findings suggesting a role for vitamin D\textsubscript{3} in benefiting immune function, inflammatory processes and transcription regulation (Hossein-Nezhad et al. 2013). Surprisingly, when identifying the pathways in which the differentially expressed genes were associated, there was no overlap in the top-scoring pathways affected between the vitamin D\textsubscript{2} and vitamin D\textsubscript{3} interventions, suggesting that the two forms of vitamin D may influence different metabolic/cellular activities. At this stage it is not clear whether the changes observed in the D\textsubscript{2}-treated subjects may have a detrimental effect on health, since they are not seen in the vitamin D\textsubscript{3}-treated subjects.

These differences in gene expression could be attributed to one of two things: firstly they could be due to the effect of 25OHD\textsubscript{2} concentrations per se on gene expression in comparison to the 25OHD\textsubscript{3} concentrations seen in the vitamin D\textsubscript{3} intervention. Alternatively, the differences seen in the vitamin D\textsubscript{2} intervention could actually result from the decrease in 25OHD\textsubscript{3} concentrations that is observed within this group, as presented graphically in Figure 6-1.

### 6.4.3 Novel Vitamin D-Responsive Genes Identified in this D2-D3 study

The genes identified within the current study: the D2-D3 Study dataset, were compared with the genes identified in previous analyses to identify common genes and also novel findings. Although some common genes were found, the great majority (> 1,000) are identified here for the first time.

It was not surprising that there was little concordance between this current D2-D3 Study dataset and previous reports due to differences in study designs. Firstly, the total sample size of Hossein-Nezhad and colleague’s study was eight subjects (three on 10 \(\mu\)g/d and five on 50 \(\mu\)g/d), and was run as a pilot study, therefore lacking sufficient statistical power to justify their conclusions. Also, the VitDmet studies (Vukic et al. 2015; Saksa et al. 2015) only selected candidate genes for analysis, whereas genome-wide analysis was used in the current study. Lastly, the \textit{in vitro} analysis (Ramagopalan et al. 2013) used the active form of vitamin D\textsubscript{3}, 1,25(OH)\textsubscript{2}D\textsubscript{3} directly on a cultured cell line, whereas the current study gave vitamin D\textsubscript{2} and vitamin\textsubscript{3} via the diet in a clinical trial setting.
6.4.4 Further Work

The analysis of the transcriptome data presented in this chapter represents just a small snapshot of the results that will become available from this dataset over time. Further in-depth analysis is ongoing.

The plans for further analysis include a more extensive examination of the differences shown in gene expression between those on the vitamin D$_2$ intervention and those on the vitamin D$_3$ intervention. Paxgene RNA tube samples were collected from participants on the placebo intervention, and so future work may include the measurement of gene expression over time in the placebo group, who similarly to the vitamin D$_2$ intervention group, showed a decrease in 25OHD$_3$ concentrations. If those on the placebo intervention had a similar gene expression pathway to those on the vitamin D$_2$ intervention group then it would be likely that decreases in 25OHD$_3$ concentrations per se is what is driving the gene expression differences seen between the vitamin D$_2$ and vitamin D$_3$ interventions, where vitamin D$_2$ supplementation is leading to a reduction in serum 25OHD$_3$ concentration.

With evidence showing ethnic minority groups, such as South Asians, are at a higher risk of vitamin D deficiency (Darling et al. 2013a), elucidating differences in gene expression between Caucasian and South Asian women at baseline and in response to supplementation may suggest mechanisms that explain lower vitamin D concentrations in the South Asian, or that vitamin D requirements to activate the same pathways are lower in South Asian populations. Differences in gene expression between the two ethnic groups - South Asian and Caucasian women - is therefore another key question to address for future work, and larger study numbers are needed to ensure this analysis would be sufficiently powered. Additional blood samples are already available for a more comprehensive analysis of ethnic differences.

As participants were selected for analysis based on their response to the intervention, one area that is to be examined is the difference in gene expression in those who were ‘responders’ to supplementation and those who were ‘non-responders’. Determining associations between gene expression and serum 25OHD concentrations would also be interesting, as 25OHD concentrations have not yet been factored into the analyses.

Our main findings will also require independent validation using quantitative real time PCR (qPCR) quantification of selected target genes.
6.4.5 Strengths

This study is the largest to study how vitamin D supplementation affects genomic expression in vivo in humans. It is also the first and only study to have compared the respective influences vitamin D$_2$ and vitamin D$_3$. The randomized-controlled trial, which formed the basis for this genomic analysis, had many strengths that have been discussed previously in Chapter 3.4.5.

Additional strengths of this study include the blood sample collection methods used. PAXgene Blood RNA Tubes were used which contain a reagent that lyses blood cells and immediately stabilises intracellular RNA, and prevents further RNA synthesis, to preserve the gene expression profile. RNA stabilisation is critical for reliable downstream gene expression analysis. Without stabilization/transcription inhibition, both degradation of RNA and up regulation or down regulation of transcripts could occur immediately after blood is drawn. This form of whole blood RNA collection offers a number of technical advantages over alternative methods of isolating the blood leukocyte compartment from whole blood through the collection of buffy coats, such as reduced laboratory processing time. Additionally the extracted mRNA samples were only taken forward for labeling and microarray use if they had a RNA Integrity Number (RIN) >7, which means the quality of samples used was reproducibly high.

The dataset also has great strengths in that participants were selected for RNA analysis based on intervention, ethnicity and response to supplementation to ensure a representative group, and to allow for future analysis comparing ethnic groups and ‘non-responders’ to ‘responders’.

6.4.6 Limitations

One current limitation of this dataset is that the numbers of the South Asian and Caucasian cohorts are not balances (22 versus 28, respectively); however suitably prepared samples from additional participants on the D2-D3 Study are stored in a -80°C freezer and participant numbers for these analyses will be increased if further grant funding is forthcoming.

6.4.7 Conclusion

This preliminary analysis of gene expression in response to vitamin D$_2$ compared with vitamin D$_3$ has provided an exciting insight into potential key differences in the metabolic/cellular response to vitamin D$_2$ and vitamin D$_3$ supplementation. Further research is however needed, to determine whether the response to vitamin D$_2$ is attributable to a decrease in 25OHD$_3$ concentrations (observed in this study following vitamin D$_2$ treatment) or whether it is in response to the increase in 25OHD$_2$ concentrations per se.
7.1 INTRODUCTION

7.1.1 Background

Vitamin D status is influenced by personal, social and environmental factors that influence sunlight exposure and diet, but genetic factors have also been shown to be important. Heritability of 25OHD concentrations has been reported to range from 28.8% (Shea et al. 2009), to 68.9% in an adolescent twin study (Arguelles et al. 2009), to 80% in a genome-wide linkage scan in asthma families (Wjst et al. 2007). A study in both monozygotic and dizygotic twins has shown that environmental variance accounts for only 30% (26-35%) of the age-adjusted heritability of 25OHD, and 35% (26-47%) of that for 1,25(OH)2D, whereas genetic variance accounts for 43% (28-57%) and 65% (53-74%), respectively (Hunter et al. 2001). Kuhn and colleagues (2014) suggested that independent of latitude there seems to be considerable variation in 25OHD, and thus they aimed to investigate the dietary, lifestyle, anthropometric and genetic determinants of this. Within the EPIC (European Prospective Investigation into Cancer and Nutrition) cohort, season was by far the strongest predictor of 25OHD (14.8%) whereas the dietary, lifestyle, anthropometric and genetic determinants that were significantly associated with 25OHD explained <2.3% of the variance. They concluded that the use of 25OHD prediction scores as a low-cost alternative to directly measuring 25OHD status were not feasible.

Although we, therefore, know that heritability of 25OHD has been shown to range from 28.8 to 80%, there are only a few genes and genetic variants that have so far been identified as being associated with 25OHD concentrations and vitamin D metabolites. Identifying genetic variants in the form of single nucleotide polymorphisms (SNPs) has been one of the key methods for identifying genetic variants associated with 25OHD status.

To date key targets for SNP searches have been the gene regions encoding for proteins and enzymes involved in the vitamin D metabolism pathway, such as the GC gene, DHCRT7, CYP2R1, CYP24A1, CYP27B1 and VDR as shown in Figure 7-1. The GC gene (group-specific component), also known as the vitamin D binding protein, transports 25OHD and 1,25(OH)2D through the two step hydroxylation process. The DHCRT7 gene encodes 7-dehydrocholesterol reductase which catalyses the conversion of 7-dehydrocholesterol to cholesterol and is involved in the pathway of vitamin D3 synthesis from sunlight exposure. CYP2R1 and CYP27B1 code for 25-hydroxylase and 1-alpha-hydroxylase, respectively, which are both key enzymes in the two step hydroxylation process, whereas CYP27B1
codes for 24-hydroxylase which is involved in the deactivation of 1,25(OH)₂D. The vitamin D receptor (VDR) is a transcription factor that is activated by the active form of vitamin D, 1,25(OH)₂D.

![Diagram of vitamin D metabolism, transportation, and signaling pathways.](https://tulane.edu/publichealth/bio/genetic-and-epigenetic-studies-on-vitamin-d.cfm)

**Figure 7-1** Metabolism, transportation, and signaling pathways of vitamin D. Taken from https://tulane.edu/publichealth/bio/genetic-and-epigenetic-studies-on-vitamin-d.cfm

However, genome-wide association studies (GWAS) have the ability to identify SNPs beyond such candidate (selected) genes from within the vitamin D pathway. To date, there have been a few GWAS that have identified gene variants associated with vitamin D status, and this is useful for identifying those who may be at risk of deficiency, and targeting treatment to reduce the impact of vitamin D deficiency.

### 7.1.2 Cross-Sectional Association between 25OHD and SNPs

In 2010, a systematic review of the associations between common SNPs and 25-hydroxyvitamin D concentrations was conducted (McGrath et al. 2010), bringing together the findings of 17 studies. Two of these were GWAS (Benjamin et al. 2007; Engelman et al. 2008) and 15 were candidate gene studies (Abbas et al. 2008; Ahn et al. 2009; d’Alesio et al. 2005; Engelman et al. 2008; Fang et al. 2009; Fu et al. 2008; Fu et al. 2009; Hyppönen et al. 2009; Kurylowicz et al. 2006; Orton et al. 2008; Ramos-Lopez et al. 2007; Ramos-Lopez et al. 2008; Sinotte et al. 2009; Smolders et al. 2009;
Smolders et al. 2009; Wjst et al. 2006). The most consistent finding that emerged was the association between rs4588 and rs7041 (both in the vitamin D binding protein/GC), or the derived haplotypes from these two SNPs, with 25OHD. Several SNPs within the vitamin D receptor (VDR) were also examined, but only one showed a consistent association in two of the three studies that examined it - rs10725810 (also known as the Fok1 polymorphism and recently merged with rs2228570). Within the cytochrome p450 genes involved in vitamin D metabolism (CYP27B1, CYP27A1, CYP24A1, CYP2R1) several SNPs were investigated across the 17 studies, but only one SNP (CYP27B1: rs10877012) showed a significant association with 25OHD in more than one study. Further isolated associations between SNPs and 25OHD concentrations were found, but were not consistent across other studies.

Since this systematic review, Bu and colleagues (2010) selected 49 SNPs from nine important genes in the vitamin D metabolism pathway and following discovery, replication and pooled analyses, found rs12794714 and rs10766197 (CYP2R1) and rs222020 (GC) remained significantly associated with 25OHD concentrations. Barry and colleagues (2014) have shown associations between 25OHD concentrations and five SNPs within the GC gene (rs12512631, rs4588, rs7041, rs222020, rs1155563), four SNPs within CYP2R1 gene (rs12794714, rs10741657, rs1562902, rs10766197) and two SNPs within CYP24A1 (rs2209314, rs2762939).

7.1.2.1 Genome-wide Association Studies

Since the systematic review conducted by McGrath and colleagues (2010), which included two GWAS, there have been two further GWAS in populations of European decent (Wang et al. 2010; Ahn et al. 2010). Both GWAS identified SNPs that were significantly associated with circulating 25OHD concentrations, unlike the two previous GWAS. The SUNLIGHT consortium (Study of Underlying Genetic Determinants of Vitamin D and Highly Related Traits) identified rs2282679 (GC), rs12785878 (DHCR7) and rs10741657 (CYP2R1) as significantly associated with 25OHD concentrations in a cohort of 33,996 adults from 15 different cohorts (Wang et al. 2010). The researchers developed genotype risk scores (GRS) based on these three SNPs and found that those within the highest quartile of the GRS were at an increased risk of having 25OHD concentrations below 50 nmol/l compared to those in the lowest quartile. Ahn and colleagues (2010) also found a significant association between rs2282679 (GC) and 25OHD concentrations, in a cohort of 4,501 adults from five different cohorts, but did not replicate any of the other associations shown by Wang and colleagues (2010). Although not significant, they did suggest a trend towards an association between 25OHD concentrations and
the following SNPs: rs7041 (GC), rs1155563 (GC), rs1790349 (DHCR7), rs3829251 (NADSYN1), rs6599638 (ACADSB), and rs2060793 (CYP2R1).

In addition to these previous GWAS that have examined associations in adulthood, Anderson and colleagues (2014) completed the first GWAS to study associations in childhood. Using the Western Australian Pregnancy cohort they looked at associations at six years of age (n=673) and 14 years of age (n=1,140). At age six, rs1007392 (CYP2R1) and rs17467825 (GC) were associated with 25OHD concentrations, whereas at age 14 rs11023332 (CYP2R1) and rs1155563 (GC) were significant.

7.1.3 SNPs and the 25OHD Response to Vitamin D Supplementation

As it has been shown that some SNPs within genes involved in the metabolism of vitamin D, such as the vitamin D binding protein (GC), are associated with 25OHD concentrations, it is plausible that these SNPs could also affect the change in 25OHD in response to vitamin D supplementation or a set dose of vitamin D. If this is the case, genetic factors may need to be taken into account when recommending dietary recommendations of vitamin D and/or vitamin D supplementation.

There are studies that have begun to examine the associations between SNPs and response to supplementation. Fu and colleagues (2009) studied the associations between rs4588 and rs7041 (GC) and change in 25OHD in response to supplementation, in 98 adults receiving either 15 µg/d (600IU/d) or 100 µg/d (4000IU/d) vitamin D₃ for one year. For rs4588 they found a significantly higher total change in 25OHD concentrations after supplementation in those with the homozygous minor genotype, who also had the lowest baseline 25OHD. This could suggest an adaptive mechanism may be in place to optimise improving 25OHD concentrations when sources (i.e. supplements) are available, but whether these are directly an effect of the genotype or simply an adaptive mechanism in place for the improvement of lower vitamin D concentrations is not clear, as baseline 25OHD concentrations were not controlled for. Another study in 285 participants following supplementation with 1,000 µg/d (40,000 IU/d) vitamin D for six months, showed the opposite. They found significant associations for rs2282679 (GC) and rs10741657 (CYP2R1) with total change in 25OHD concentrations, in both cases showing a higher increase in those within the major homozygous allele who had the highest baseline 25OHD (Didriksen et al. 2013). Following 1 year of supplementation with 25 µg/d (1,000 IU/d) vitamin D₃, calcium carbonate, both or placebo, Barry and colleagues (2014) identified rs10766197 (CYP2R1), rs6013897 (CYP24A1) and rs7968585 (VDR) as
SNPs that modify 25OHD response to supplementation, when controlling for baseline 25OHD concentrations, but did not find significance for those SNPs identified previously.

As discussed in Chapter 1.9, there is controversy as to the efficacy of vitamin D$_2$ vs. D$_3$ in raising total 25OHD concentrations. However, following the results presented in Chapter 3, vitamin D$_3$ is more effective than vitamin D$_2$. To date, there has been no published works investigating whether genetic variants known to affect homeostatic concentrations of 25OHD have any impact on the change in 25OHD in response to vitamin D$_2$ vs. vitamin D$_3$ supplementation.

7.1.4 Ethnic Variation

There is evidence to show that different ethnicities, in particular South Asians, have higher rates of vitamin D deficiency as discussed in Chapter 1.6.1 (Macdonald et al. 2011, Darling et al. 2013a). Despite the differences in 25OHD concentrations seen between ethnic groups, the majority of the research examining associations between SNPs and 25OHD concentrations has been focused on Caucasian populations. Very limited research has been completed in the South Asian population, particularly UK dwelling, and so the contribution of genetics to the differences in vitamin D status cannot be quantified.

Allele frequencies of common genetic variants have been shown to differ between ethnic groups, which accounts for differences in gene expression among these groups (Spielman et al. 2007). Of particular interest, in relation to the differing prevalence of vitamin D deficiency between ethnic groups, is the differences shown between ethnic groups for genetic variants within the GC gene and the CYP genes (Kamboh & Ferell, 1986), which may have an impact on the metabolism of vitamin D between ethnic groups.

There are limited studies that have studied the association between SNPs and 25OHD concentrations between ethnic groups. One study comparing African Americans (n=652) and Caucasian Americans (n=405) identified distinct differences in the SNP associations with 25OHD concentrations. The SNP with the strongest association with 25OHD differed between the two ethnic groups; rs12794714 (CYP2R1) in African Americans and rs1993116 (CYP2R1) in Caucasian Americans (Batai et al. 2014). Interestingly though, the same gene (CYP2R1) is identified in both ethnic groups. Elkum and colleagues (2014) have looked at Arab (n=907), South Asian (n=489) and Southeast Asian (n=153) populations and identified three SNPs that are exclusively associated with 25OHD concentrations.
within the Arab population only (GC gene: rs1155563, CYP2R1: rs10500804 and rs12794714), one SNP that is exclusive to the South Asian population (CYP2R1: rs10741657), and five SNPs that are associated with 25OHD concentrations in both Arab and South Asian populations (GC gene: rs17467825, rs2282679, rs3755967, rs2298850 and rs7041). Of the 18 SNPs included within this study, none were associated with 25OHD concentrations in the Southeast Asian population. Suaini and colleagues (2014) investigated genetic and environmental determinants of vitamin D insufficiency in 12-month old infants in both a Caucasian population (n=491) and an Asian (East Asian) population (n=72). Within this study the researchers identified several SNPs within the GC and VDR that were associated with 25OHD concentrations only in the Caucasian group: rs1155563, rs17467825, rs2282679, rs2298849, rs3755967, rs4588 and rs7041 in GC; and rs2239185 and rs7975232 in VDR. However, this study may have lacked power to assess the relevance of genetic variants in the Asian population.

These differences between ethnic groups provide an initial insight into the potential contribution of genetics to the variation in vitamin D status between ethnic groups. This highlights the potential need for deficiency screening to be targeted at ‘at risk’ groups, and suggests that optimal treatments may vary between the groups. However, there is currently limited evidence examining the effect of genetic variants on response to supplementation, within minority ethnic groups.

7.1.5 Vitamin D Binding Protein

The vitamin D binding protein (VDBP) binds to vitamin D and its metabolites, and transports them to target tissues. Moy and colleagues (2014) aimed to characterise SNPs that may influence concentrations of VDBP, rather than 25OHD concentrations, via a GWAS approach. They identified two SNPs in the GC gene that associated negatively with VDBP concentrations. In the case of rs7041, those with two copies of the minor allele (TT) had a VDBP concentration that was 57% lower than those with no copies of the minor allele. A study by Powe and colleagues (2013) found that black Americans were more likely to have SNPs within the GC gene, associated with lower VDBP concentrations and also lower 25OHD$_3$ concentrations, compared to the Caucasian Americans. However, as they saw a parallel decrease in 25OHD and VDBP concentrations within the black American group, they suggested that this parallel relationship is likely to result in a similar level of bioavailable vitamin D to the Caucasian group, despite differences in 25OHD concentrations. This suggests that certain ethnic groups that have lower 25OHD concentrations may also have a higher
frequency of polymorphisms within the VDBP gene (GC gene) that result in lower VDBP concentrations, which may be beneficial to vitamin D bioavailability.

7.1.6 Bone Mineral Density

Vitamin D, through its active form (\(1,25(\text{OH})_2\text{D}\)) plays a crucial role in bone metabolism. Genetic variants within the VDR have previously been shown to be associated with bone mineral density (BMD) in the lumbar spine, in patients with primary hyperparathyroidism (Christensen et al. 2013) and osteoporotic women (Dundar et al. 2009). However, in a meta-analysis of studies across Europe, genetic polymorphisms within the VDR where not associated with BMD (Uitterlinden et al. 2006).

7.1.7 Justification for Study

Understanding how genetic variants, such as SNPs, influence 25OHD concentrations, VDBP concentrations, bone mineral density, and response to vitamin D\(_2\) and D\(_3\) supplementation could prove useful for the identification of ‘at risk’ groups and successful management of vitamin D deficiency and/or insufficiency. If 25OHD concentrations are associated with a genotype, then some individuals may be at a higher risk of vitamin D deficiency or insufficiency. They may also require earlier treatment to prevent deficiency or insufficiency, or may need to be monitored more regularly. If response to supplementation with vitamin D\(_2\) or D\(_3\) is differentially associated with genotype this would be crucial in the treatment of deficiency, in identifying those who may need a higher dose or a particular form of vitamin D (D\(_2\) or D\(_3\)) for optimal increase in 25OHD. Policy makers would also find this information key, as it would suggest that a ‘one size fits all’ mandatory fortification scheme or recommended daily amount may not be best practice.

7.1.8 Aims and Objectives of Study

**Aim 1** – To determine whether SNPs within genes with functions in the metabolism of vitamin D, are associated with serum 25OHD concentrations, VDBP, or volumetric BMD (vBMD), in both South Asian and Caucasian women.

**Hypothesis** – The selected SNPs, within genes relevant to vitamin D metabolism, would be associated with 25OHD concentrations, in both the South Asian and Caucasian women. In addition, SNPs within the GC gene would be associated with VDBP concentrations, and SNPs
within the VDR gene would be associated with vBMD, in both the South Asian and Caucasian women.

**Aim 2** – To determine whether the SNPs, within genes relevant to vitamin D metabolism, are associated with response to 15 µg/d (600 IU/d) vitamin D₂ vs. vitamin D₃, in both South Asian and Caucasian women.

**Hypothesis** – The selected SNPs, within genes relevant to vitamin D metabolism, would be associated with response to both vitamin D₂ and vitamin D₃ supplementation (change in 25OHD concentration), in both the South Asian and Caucasian women.
7.2 METHODS

7.2.1 Study Cohorts/Participants

Stored samples from two cohorts of subjects were used in these analyses: subjects from the D-FINES Study (described below) and the D2-D3 Genetic Study (described in Chapter 2.1). The D-FINES Study formed a discovery cohort and the D2-D3 Genetic Study formed the replication cohort (to validate any findings from the discovery cohort analysis) used in the analyses investigating associations between 25OHD concentrations and genotype. The D2-D3 Study subjects were also used in analyses examining associations between genetic variants and VDBP and vBMD, as well as the analyses identifying associations between genotype and change in 25OHD concentrations, following a randomised-controlled trial detailed in Chapter 2.1. Both cohorts included adults from two ethnic group; South Asian and Caucasian.

7.2.1.1 D-FINES Study

The D-FINES Study was a longitudinal study that was completed between 2006 and 2007, and designed to investigate the interaction between diet and sunlight exposure on vitamin D status and functional markers of bone health in pre- and post-menopausal Caucasian and South Asian women living in Southern England at (51°N). The study had ethical approval from the University of Surrey Ethics Committee (EC/2006/19/SBMS) and NHS South-West Surrey Local Research Ethics Committee (06/Q1909/1). All participants had completed a consent form (Appendix 43). Data were collected at four time points; Summer 06, Autumn 06, Winter 06/07 and Spring 07. Data collected included anthropometric measurements and fasted blood samples for 25OHD concentrations. Serum 25OHD concentrations were measured using the manual Immunodiagnostics Systems (IDS) enzyme immunoassay (Immunodiagnostic Systems Ltd, Bolton, Tyne and Wear, UK) at a DEQAS approved laboratory (Dr Jacqueline Berry, University of Manchester). Manufacturer’s reference ranges were 48-144 nmol/L (19-58 ng/ml); sensitivity 5 nmol/L (2 ng/ml); intra- and inter- assay coefficients of variation (CV) 6% and 7% respectively.

7.2.2 Candidate SNPs

Fourteen SNPs from six genes were selected according to the following criteria:
(1) evidence of significant association with vitamin D status in GWAS and candidate gene studies
(2) within genes of biological importance in vitamin D metabolism, transportation, degradation, or downstream signaling activation.

The selected SNPs, basic characteristics, and justification for inclusion are shown in Table 7.1.
Table 7.1 Characteristics of single nucleotide polymorphisms (SNPs) selected for inclusion in analyses

<table>
<thead>
<tr>
<th>Nearest Gene(s) Acronym</th>
<th>SNP</th>
<th>M/m</th>
<th>Risk Allele</th>
<th>Chromosome</th>
<th>Location</th>
<th>Nearest Gene(s) Full Name</th>
<th>Protein Coded and/or Function Related to Vit D</th>
<th>Prior Evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>rs4588</td>
<td>C/A</td>
<td>A</td>
<td>4</td>
<td>71752617</td>
<td>group-specific component (vitamin D binding protein)</td>
<td>Vitamin D binding protein - binds to vitamin D and its metabolites and transport them to target tissues</td>
<td>[1-2] [3-4]</td>
</tr>
<tr>
<td></td>
<td>rs7041</td>
<td>G/T</td>
<td>T</td>
<td>71752617</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHCR7</td>
<td>rs12785878</td>
<td>T/G</td>
<td>G</td>
<td>11</td>
<td>71456403</td>
<td>7-dehydrocholesterol reductase</td>
<td>7-dehydrocholesterol reductase - catalyses the conversion of 7-dehydrocholesterol to cholesterol</td>
<td>[1][4] [4] [3][5]</td>
</tr>
<tr>
<td></td>
<td>rs4944957</td>
<td>G/A</td>
<td>A</td>
<td></td>
<td>71456989</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs11234027</td>
<td>G/A</td>
<td>A</td>
<td></td>
<td>71253061</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2R1</td>
<td>rs10741657</td>
<td>G/A</td>
<td>G</td>
<td>11</td>
<td>14893332</td>
<td>cytochrome P450, family 2, subfamily R, polypeptide 1</td>
<td>25 hydroxylase - hydroxylation of vitamin D to 25OHD in the liver</td>
<td>[1][4] [4][6]</td>
</tr>
<tr>
<td></td>
<td>rs12794714</td>
<td>G/A</td>
<td>A</td>
<td></td>
<td>14892029</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP24A1</td>
<td>rs6013897</td>
<td>T/A</td>
<td>A</td>
<td>20</td>
<td>54125940</td>
<td>cytochrome P450, family 24, subfamily A, polypeptide 1</td>
<td>24-hydroxylase - deactivation of 1,25OH$_2$D</td>
<td>[1-2]</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>rs10877012</td>
<td>G/T</td>
<td>T</td>
<td>12</td>
<td>57768302</td>
<td>cytochrome P450, family 27, subfamily B, polypeptide 1</td>
<td>1-alpha-hydroxylase: activates 25OHD in the kidneys and other organs</td>
<td>[2][6]</td>
</tr>
<tr>
<td>VDR</td>
<td>rs2228570</td>
<td>C/T</td>
<td>C</td>
<td>12</td>
<td>47849112</td>
<td>vitamin D receptor</td>
<td>Represses expression of 1-alpha-hydroxylase and induces expression of CYP24A1, which deactivates 1,25OH$_2$D</td>
<td>[6] [6] [6][6] [6]</td>
</tr>
<tr>
<td></td>
<td>rs1544410</td>
<td>G/A</td>
<td>A</td>
<td></td>
<td>47846052</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs731236</td>
<td>T/C</td>
<td>C</td>
<td></td>
<td>47844974</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs11568820</td>
<td>G/A</td>
<td>A</td>
<td></td>
<td>47908762</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs7975232</td>
<td>A/C</td>
<td>C</td>
<td></td>
<td>47845054</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

SNP = single nucleotide polymorphism, M/m = major/minor allele, a the allele associated with a lower 25OHD concentration from prior evidence, b Chromosomal location based on Genome Reference Consortium Human Build 38 patch release 2 (GRCh38.p2).

7.2.3 DNA extraction and SNP Genotyping

DNA extraction and SNP genotyping were completed by LGC Genomics (Herts, UK). DNA was extracted from whole blood following their DNA extraction protocol (Appendix 44), and SNP genotyping was completed using the KASP assay - a homogeneous fluorescent genotyping system based on competitive allele-specific polymerase chain reaction (PCR) (Appendix 45). For quality control purposes, non-template controls (NTC) were used.

7.2.4 Statistical Genetic Analysis

The two datasets were checked independently for basic information on all SNPs, including call rate per SNP and genotype frequencies, and were tested for deviation from Hardy-Weinberg Equilibrium to check that allele frequencies in the study population were consistent with previous populations. Frequencies from the International HapMap Project were used for comparison (The International HapMap Consortium 2003). The datasets were coded based on the allele associated with risk of lower vitamin D concentrations from previous evidence, as indicated in Table 7.1. The higher the number, the greater the risk of low vitamin D concentrations.

All analyses were performed with the use of SPSS * software (version 22.0; SPSS Inc., Chicago, IL). Differences were considered to be significant at p-value <0.05. However, if more stringent testing were applied a p-value of <0.008 would be more appropriate due to multiple testing (0.05 ÷ 6 (number of genes included) = 0.008), and so p-values <0.008 have been identified throughout. Data were checked for normality using the Kolmogorov-Smirnov test. Pearson (or Spearman where data was not normally distributed) correlations were used to identify associations between age and BMI, with 25OHD or percentage change in 25OHD, to check whether age and BMI are potential confounders. Linear regression, controlling for relevant confounders, was then used to determine associations between genotypes and outcomes: 25HOD concentrations and percentage change in 25OHD concentrations. Results are presented as mean values and standard deviations (SD) in the text and tables, and as mean values and standard error of the mean (SEM) in graphs.
7.3 RESULTS

7.3.1 Genotyping

All 14 SNPs were successfully genotyped, and mean genotyping call rates were 99.7% in the participants within the D2-D3 Genetic Study and 99.2% in the participants within the D-FINES Study. For quality control, 1% of samples were duplicated and the reproducibility was 100%.

Genotype frequencies and the minor allele frequency (MAF) for the two ethnic groups, in both cohorts are given in Table 7.2, alongside the MAF from the International HapMap Project, Caucasian European dataset for comparison. Within ethnic groups combined within each study cohort, only two SNPs showed deviation from the Hardy-Weinburg equilibrium ($\chi^2 \geq 3.841$) in both the discovery and replication cohorts, suggesting that subject selection and genotyping was relatively unbiased. The two SNPs that did show deviation from the Hardy-Weinburg equilibrium were rs12785878 and rs4944957 of the DHCR7 gene. When split by ethnicity, as shown in Table 7.2, the MAF for those two SNPs differ between ethnic groups, with the Caucasian MAF of the discovery and replication cohorts similar to that of the HapMap CEU. However, there were no data available in the HapMap Project from a South Asian cohort for comparison, and therefore these SNPs were not excluded from the analyses.
Table 7.2 Genotype frequency and minor allele frequency (MAF) for each SNP, within ethnic groups of the discovery cohort and the replication cohort, and the MAF of the International HapMap Project CEU dataset.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Discovery Cohort</th>
<th>Replication Cohort</th>
<th>HapMap CEU1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MM/Mm/mm</td>
<td>South Asian</td>
<td>Caucasian</td>
</tr>
<tr>
<td>GC</td>
<td>CC/CA/AA</td>
<td>50/30/1</td>
<td>130/99/23</td>
</tr>
<tr>
<td>rs4588</td>
<td>MAF</td>
<td>0.198</td>
<td>0.288</td>
</tr>
<tr>
<td>rs7041</td>
<td>GG/GT/TT</td>
<td>27/36/19</td>
<td>77/120/55</td>
</tr>
<tr>
<td>DHCRT</td>
<td>MAF</td>
<td>0.451</td>
<td>0.456</td>
</tr>
<tr>
<td>rs12785878</td>
<td>TT/GG/TT</td>
<td>5/27/43</td>
<td>148/93/12</td>
</tr>
<tr>
<td>rs4944957</td>
<td>MAF</td>
<td>0.753</td>
<td>0.231</td>
</tr>
<tr>
<td>rs11234027</td>
<td>GG/GA/AA</td>
<td>36/34/12</td>
<td>186/64/4</td>
</tr>
<tr>
<td>CYP2R1</td>
<td>MAF</td>
<td>0.354</td>
<td>0.142</td>
</tr>
<tr>
<td>rs10741657</td>
<td>GG/GA/AA</td>
<td>31/38/11</td>
<td>94/114/44</td>
</tr>
<tr>
<td>rs12794714</td>
<td>MAF</td>
<td>0.370</td>
<td>0.401</td>
</tr>
<tr>
<td>CYP24A1</td>
<td>TT/TA/AA</td>
<td>47/24/11</td>
<td>164/75/15</td>
</tr>
<tr>
<td>rs6013897</td>
<td>MAF</td>
<td>0.280</td>
<td>0.207</td>
</tr>
<tr>
<td>CYP27B1</td>
<td>GG/GT/TT</td>
<td>24/40/18</td>
<td>126/99/28</td>
</tr>
<tr>
<td>VDR</td>
<td>MAF</td>
<td>0.463</td>
<td>0.306</td>
</tr>
<tr>
<td>rs2228570</td>
<td>CC/CT/TT</td>
<td>45/33/4</td>
<td>101/116/37</td>
</tr>
<tr>
<td>rs1544410</td>
<td>MAF</td>
<td>0.250</td>
<td>0.374</td>
</tr>
<tr>
<td>rs731236</td>
<td>GG/GA/AA</td>
<td>26/44/12</td>
<td>100/111/43</td>
</tr>
<tr>
<td>rs11568820</td>
<td>MAF</td>
<td>0.415</td>
<td>0.388</td>
</tr>
<tr>
<td>rs7975232</td>
<td>TT/TC/CC</td>
<td>36/35/10</td>
<td>101/110/43</td>
</tr>
<tr>
<td>VDR</td>
<td>MAF</td>
<td>0.340</td>
<td>0.386</td>
</tr>
<tr>
<td>rs2228570</td>
<td>GG/GA/AA</td>
<td>39/34/9</td>
<td>163/80/11</td>
</tr>
<tr>
<td>rs11568820</td>
<td>MAF</td>
<td>0.317</td>
<td>0.201</td>
</tr>
<tr>
<td>rs7975232</td>
<td>AA/AC/CC</td>
<td>26/37/17</td>
<td>67/112/72</td>
</tr>
<tr>
<td></td>
<td>MAF</td>
<td>0.444</td>
<td>0.510</td>
</tr>
</tbody>
</table>

M=major allele, m=minor allele, MM=major homozygous, Mm=heterozygous, mm=minor homozygous, MAF=minor allele frequency, 1MAF from the NCBI HapMap CEU (Caucasian European) database
7.3.2 Participant Characteristics

7.3.2.1 Discovery Cohort

The discovery analyses were completed using data and samples collected from the D-FINES Study, consisting of 82 South Asian and 254 Caucasian women. As shown in Table 7.3, the South Asian cohort had a mean 25OHD concentration of 22.80 ±11.28 nmol/L, mean age of 47.52 ±13.30 years and mean BMI of 27.79 ±5.41 kg/m². The Caucasian cohort had a mean 25OHD concentration of 48.26 ±20.50 nmol/L, mean age of 48.27 ±14.49 years and mean BMI of 26.10 ±14.49 kg/m².

7.3.2.2 Replication Cohort

Out of a possible total of 90 South Asian and 234 Caucasian women recruited as part of the D2-D3 Main Study, 81 South Asian and 234 Caucasian women gave consent to be part of the D2-D3 Genetic Study. As shown in Table 7.3, the South Asian cohort had a mean 25OHD concentration of 28.60 ±22.86 nmol/L, mean age of 37.65 ±10.35 years and a mean BMI of 25.44 ±4.39 kg/m². The Caucasian cohort had a mean 25OHD concentration of 60.05 ±26.33 nmol/L, mean age of 45.58 ±12.31 years and a mean BMI of 23.61 ±3.44 kg/m².

<table>
<thead>
<tr>
<th>Table 7.3 Participant characteristics of both the discovery and replication cohorts, stratified by ethnicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Discovery Cohort</td>
</tr>
<tr>
<td>South Asian</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Age (yrs)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>25OHD (nmol/L)</td>
</tr>
</tbody>
</table>

Key: BMI = body mass index, discovery cohort = D-FINES Study data, replication cohort = D2-D3 Study data.
7.3.2.3 **D2-D3 RCT Cohort**

Out of a possible total of 81 South Asian and 234 Caucasian women who consented to the D2-D3 Genetic Study, 55 South Asians and 218 Caucasian women successfully completed the D2-D3 Study 12-week protocol and were therefore used in these analyses.

Mean age, BMI, 25OHD concentration and change in 25OHD for each treatment group are shown in Table 7.4. Within each ethnic group there were no significant differences between the three treatment groups for age, BMI or 25OHD concentration. However, in the South Asian women the vitamin D₃ group had a significantly higher percentage change in 25OHD than both the placebo (p<0.001) and the vitamin D₂ group (p=0.020), and in the Caucasian women all three groups had a significantly different percentage change in 25OHD (p<0.002 in all cases). Between ethnic groups, the Caucasian 25OHD concentrations were all significantly higher than all of the South Asian 25OHD concentrations (p<0.008), and BMI in the South Asian vitamin D₂ group was significantly higher than the Caucasian vitamin D₃ group (p=0.033). The percentage change in 25OHD was also significantly higher in the South Asian vitamin D₂ cohort than the Caucasian placebo and vitamin D₂ groups (p<0.020 in both cases), and the South Asian vitamin D₃ group had a significantly higher percentage change than all three Caucasian groups (p<0.001 in all cases).

Table 7.4 Participant Characteristics from the D2-D3 Study RCT Cohort, stratified by ethnicity and intervention group

<table>
<thead>
<tr>
<th></th>
<th>Placebo</th>
<th>South Asian</th>
<th>Caucasian</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vitamin D₂</td>
<td>Vitamin D₃</td>
</tr>
<tr>
<td>n</td>
<td>11</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>40.45 ±9.03</td>
<td>39.42 ±10.27</td>
<td>41.15 ±10.56</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.81 ±2.75</td>
<td>25.84 ±4.93</td>
<td>25.22 ±4.48</td>
</tr>
<tr>
<td>25OHD (nmol/L)</td>
<td>23.87 ±11.36</td>
<td>34.26 ±23.08</td>
<td>29.21 ±20.76</td>
</tr>
<tr>
<td>Change in 25OHD (%)</td>
<td>-8.7 ±41.2</td>
<td>104.0 ±108.5</td>
<td>231.9 ±217.9</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>88</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>45.45 ±11.97</td>
<td>45.49 ±12.48</td>
<td>45.78 ±12.27</td>
</tr>
<tr>
<td></td>
<td>24.44 ±3.70</td>
<td>23.63 ±3.33</td>
<td>23.32 ±3.32</td>
</tr>
<tr>
<td></td>
<td>58.38 ±22.88</td>
<td>61.68 ±30.64</td>
<td>59.70 ±24.46</td>
</tr>
<tr>
<td></td>
<td>-25.2 ±13.3</td>
<td>38.7 ±57.3</td>
<td>72.4 ±86.4</td>
</tr>
</tbody>
</table>

BMI = body mass index, 25OHD = 25-hydroxyvitamin D

7.3.3 **GC Gene Results**

7.3.3.1 **Discovery and Replication Findings**

In both the discovery and replication cohorts, there were no significant associations identified between 25OHD concentrations and genotypes of rs4588 or rs7041, in either the South Asian or Caucasian women (data not shown).
7.3.3.2 *Vitamin D RCT Cohort*

There was a significant association between percentage change in 25OHD and genotype in the South Asian cohorts assigned the placebo intervention for both rs4588 and rs7041 genotypes, but no associations within other intervention groups (Table 7.5, Figure 7-2 and Figure 7-3) (data for the Caucasian cohort not shown).

Table 7.5 Baseline and percentage change in 25OHD in individual SNPs within the GC gene, and associations between SNP and percentage change in 25OHD in South Asian women in the D2-D3 Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethnicity</th>
<th>Treatment Group</th>
<th>Allele</th>
<th>n</th>
<th>Baseline 25OHD (nmol/L)</th>
<th>% Change in 25OHD</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC</td>
<td>South Asian</td>
<td>Placebo</td>
<td>CC</td>
<td>6</td>
<td>31.4 ±9.68</td>
<td>-31.38 ±27.2</td>
<td>r² 0.403 Beta 0.635 p-value 0.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CA</td>
<td>5</td>
<td>14.84 ±4.30</td>
<td>18.64 ±40.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D2</td>
<td>CC</td>
<td>11</td>
<td>34.64 ±27.14</td>
<td>132.6 ±132.3</td>
<td>r² 0.09 Beta -0.300 p-value 0.154</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA</td>
<td>10</td>
<td>34.81 ±21.28</td>
<td>93.56 ±83.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>3</td>
<td>34.70 ±20.10</td>
<td>33.67 ±61.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D3</td>
<td>CC</td>
<td>11</td>
<td>26.45 ±21.50</td>
<td>294.87 ±248.7</td>
<td>r² 0.107 Beta -0.328 p-value 0.158</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CA</td>
<td>9</td>
<td>32.58 ±20.55</td>
<td>154.9 ±152.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>0</td>
<td>33.52 ±23.22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7041</td>
<td>South Asian</td>
<td>Placebo</td>
<td>GG</td>
<td>4</td>
<td>36.28 ±6.93</td>
<td>-45.95 ±12.5</td>
<td>r² 0.516 Beta 0.718 p-value 0.013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GT</td>
<td>7</td>
<td>16.79 ±5.48</td>
<td>12.67 ±13.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TT</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D2</td>
<td>GG</td>
<td>5</td>
<td>34.62 ±29.86</td>
<td>105.20 ±115.3</td>
<td>r² 0.000 Beta -0.007 p-value 0.976</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT</td>
<td>10</td>
<td>34.55 ±21.46</td>
<td>104.01 ±91.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>9</td>
<td>33.73 ±23.82</td>
<td>103.27 ±133.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Vitamin D3</td>
<td>GG</td>
<td>6</td>
<td>21.60 ±16.96</td>
<td>287.40 ±210.5</td>
<td>r² 0.056 Beta -0.236 p-value 0.316</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT</td>
<td>6</td>
<td>34.73 ±23.22</td>
<td>259.77 ±301.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>8</td>
<td>30.76 ±22.37</td>
<td>169.33 ±156.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Key: GC: group-specific component (vitamin D binding protein), blue shows p<0.005
7.3.3.2.1 rs4588

There was a significant association between genotypes within the rs4588 SNP and percentage change in 25OHD concentrations, within the South Asian cohort assigned the placebo intervention ($r^2 = 0.403$, $p=0.036$) (Figure 7-2). Those with the CC genotype ($n=6$) showed a greater decrease in 25OHD concentration than those with CA genotype ($n=5$), who actually showed a mean percentage increase of 19%.

Figure 7-2 Percentage change in 25OHD concentration across genotypes of rs4588, split by intervention group and ethnicity.
Mean [$\pm$ SEM]. Linear regression $r^2$ values are shown where significant *$p<0.05$
7.3.3.2.2  rs7041

Genotypes within the rs7041 SNP were also significantly associated with percentage change in 25OHD concentrations within the South Asian cohort assigned the placebo intervention (r²=0.516, p=0.013) (Figure 7-3). Those with the GG genotype (n=4) showed a greater decrease in 25OHD concentrations than those with GT genotype (n=7), who actually showed a mean percentage increase of 13%.

Figure 7-3 Percentage change in 25OHD concentration across genotypes of rs7041, within each intervention group split by ethnicity.
Mean [± SEM]. Linear regression r² values are shown where significant *p<0.05.
7.3.4  **DHCR7 Gene**

### 7.3.4.1  Discovery and Replication Findings

There were significant associations between genotype and 25OHD concentrations within rs12785878, rs4944957 and rs11234027, in both the discovery and replication cohorts (Table 7.6).

**Table 7.6** 25OHD concentrations of individual SNPs in the **DHCR7** gene in South Asian and Caucasian women, and associations between SNP and 25OHD in both the discovery and replication cohort

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethn</th>
<th>Al</th>
<th>n</th>
<th>Mean ±SD</th>
<th>Linear Regression</th>
<th>25OHD (nmol/l)</th>
<th>Linear Regression</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs12785878</td>
<td>South Asian</td>
<td>TT</td>
<td>5</td>
<td>34.48 ±21.05</td>
<td>0.071 0.021</td>
<td>D-FINES Study</td>
<td>7</td>
<td>26.3 ±18.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG</td>
<td>27</td>
<td>23.93 ±10.84</td>
<td>-0.266</td>
<td></td>
<td>28</td>
<td>30.43 ±26.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>43</td>
<td>21.07 ±9.73</td>
<td></td>
<td></td>
<td>46</td>
<td>27.84 ±21.61</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>TT</td>
<td>148</td>
<td>49.33 ±21.25</td>
<td>0.008 0.145</td>
<td>D-FINES Study</td>
<td>138</td>
<td>62.76 ±26.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG</td>
<td>93</td>
<td>46.95 ±19.48</td>
<td>-0.092</td>
<td></td>
<td>79</td>
<td>57.90 ±27.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG</td>
<td>12</td>
<td>40.83 ±10.45</td>
<td></td>
<td></td>
<td>17</td>
<td>47.97 ±20.75</td>
</tr>
<tr>
<td>rs4944957</td>
<td>South Asian</td>
<td>GG</td>
<td>5</td>
<td>34.48 ±21.05</td>
<td>0.061 0.026</td>
<td>D-FINES Study</td>
<td>7</td>
<td>26.30 ±18.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>28</td>
<td>23.55 ±10.82</td>
<td>-0.246</td>
<td></td>
<td>31</td>
<td>31.97 ±26.72</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>49</td>
<td>21.18 ±9.73</td>
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<td></td>
<td>43</td>
<td>26.54 ±20.57</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>GG</td>
<td>149</td>
<td>49.32 ±21.18</td>
<td>0.007 0.197</td>
<td>D-FINES Study</td>
<td>138</td>
<td>62.76 ±26.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>93</td>
<td>47.52 ±20.29</td>
<td>-0.081</td>
<td></td>
<td>79</td>
<td>57.90 ±27.11</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>12</td>
<td>40.83 ±10.45</td>
<td></td>
<td></td>
<td>17</td>
<td>47.97 ±20.75</td>
</tr>
<tr>
<td>rs11234027</td>
<td>South Asian</td>
<td>GG</td>
<td>36</td>
<td>25.91 ±12.59</td>
<td>0.086 0.008</td>
<td>D-FINES Study</td>
<td>39</td>
<td>31.44 ±26.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>34</td>
<td>21.81 ±10.53</td>
<td>-0.293</td>
<td></td>
<td>35</td>
<td>25.25 ±18.06</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>12</td>
<td>16.28 ±4.31</td>
<td></td>
<td></td>
<td>7</td>
<td>29.50 ±22.95</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>GG</td>
<td>186</td>
<td>48.50 ±20.85</td>
<td>0.001 0.586</td>
<td>D-FINES Study</td>
<td>167</td>
<td>61.74 ±25.80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA</td>
<td>64</td>
<td>48.20 ±19.88</td>
<td>-0.034</td>
<td></td>
<td>62</td>
<td>57.07 ±27.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA</td>
<td>4</td>
<td>37.90 ±13.92</td>
<td></td>
<td></td>
<td>5</td>
<td>40.42 ±9.30</td>
</tr>
</tbody>
</table>

Key: Ethn: ethnicity, Al: Allele, Cauc: Caucasian, **DHCR7**: 7-dehydrocholesterol reductase, **blue** shows p<0.005, **red** shows p<0.008
7.3.4.1.1 *rs1278578*

There was a significant negative association between rs1278578 genotypes and 25OHD concentrations in the South Asian cohort in the discovery cohort (r²=0.071, p=0.021), with the vitamin D risk allele (G) being associated with lower 25OHD concentrations. However, there was no significant association in the replication cohort (Figure 7-4). In the Caucasian cohort there was no association in the discovery cohort, but there was a significant negative correlation within the replication cohort (r²=0.023, p=0.022) (Figure 7-4), with the vitamin D risk allele (G) being associated with lower 25OHD concentrations.

*Figure 7-4 Scatterplots showing associations between rs1278578 genotype and total 25OHD concentration in the discovery and replication cohorts, in South Asian and Caucasian women*

Linear regression in the discovery and replication cohorts: South Asian (r²= 0.071* and r²=0.001 respectively) and Caucasian (r²= 0.008 and r²=0.023* respectively) women. Significant correlations indicated by asterisks, *p<0.05*
7.3.4.1.2  rs4944957

There was a significant negative correlation between rs4944957 genotypes and 25OHD concentrations in the South Asian cohort in the discovery cohort \((r^2=0.061, p=0.026)\) (Figure 7-5), with the vitamin D risk allele \((A)\) being associated with a lower 25OHD concentrations, however, there was no association in the replication cohort. In the Caucasian cohort there was no association in the discovery cohort, but there was a significant negative correlation within the replication cohort \((r^2=0.023, p=0.022)\) (Figure 7-5), with the vitamin D risk allele \((A)\) being associated with lower 25OHD concentrations.

![Figure 7-5 Scatterplots showing association between rs4944957 genotype and total 25OHD concentrations in the discovery and replication cohorts, in South Asian and Caucasian women.](image)

Linear regression in the discovery and replication cohort: South Asian \((r^2=0.061^* \text{ and } r^2=0.004 \text{ respectively})\) and Caucasian \((r^2=0.007 \text{ and } r^2=0.023^* \text{ respectively})\) women. Significant correlations indicated by asterisks, *\(p<0.05\)
7.3.4.1.3 rs11234027

There was a significant negative correlation between rs112344027 genotypes and 25OHD concentrations in the South Asian cohort in the discovery cohort \((r^2=0.086, \ p=0.008)\) (Figure 7-6), with the vitamin D risk allele (A) being associated with a lower 25OHD concentrations. However, there was no association in the replication cohort. In the Caucasian group, there was no association between rs112344027 genotype and 25OHD concentrations in either the discovery or replication cohort.

![Figure 7-6 Scatterplot showing association between rs11234027 genotype and total 25OHD concentrations in the discovery and replication cohorts, in South Asian women](image)

Linear regression in discovery and replication cohorts: South Asian women \((r^2=0.086^{**} \text{ and } r^2=0.008\) respectively) . Significant correlations indicated by asterisks, \(**p<0.008\)

7.3.4.2 Vitamin D RCT Cohort

There was a significant association between SNP alleles and percentage change in 25OHD concentrations, within intervention groups, for rs12785878 and rs4944957 in the Caucasian women, but not in the South Asian (Table 7.7, data for South Asian cohort not shown). There were significant associations for rs11234027 in both the Caucasian and South Asian women (Table 7.7).
Table 7.7 Baseline and percentage change in 25OHD concentrations in individual SNPs within the **DHCR7** gene, and associations between SNP and percentage change in 25OHD concentrations in South Asian and Caucasian women in the D2-D3 Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethnic Group</th>
<th>Al</th>
<th>n</th>
<th>Baseline 25OHD (nmol/L) Mean ±SD</th>
<th>% Change in 25OHD Mean ±SD</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>r^2</td>
</tr>
<tr>
<td><strong>DHCR7</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12785878</td>
<td></td>
<td>Caul</td>
<td>TT</td>
<td>22</td>
<td>54.74 ±21.69</td>
<td>-22.22 ±11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>18</td>
<td>63.33 ±23.62</td>
<td>-27.63 ±15.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>3</td>
<td>56.63 ±30.99</td>
<td>-32.90 ±4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D2</td>
<td>TT</td>
<td>58</td>
<td>63.54 ±30.64</td>
<td>38.21 ±62.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>25</td>
<td>58.82 ±32.06</td>
<td>39.37 ±45.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>5</td>
<td>54.32 ±26.63</td>
<td>41.26 ±55.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D3</td>
<td>TT</td>
<td>47</td>
<td>66.52 ±22.63</td>
<td>51.73 ±49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>33</td>
<td>53.71 ±25.14</td>
<td>95.47 ±119.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GG</td>
<td>6</td>
<td>39.27 ±15.61</td>
<td>107.47 ±55.8</td>
</tr>
<tr>
<td>rs4944957</td>
<td></td>
<td>Caul</td>
<td>TT</td>
<td>23</td>
<td>54.74 ±21.69</td>
<td>-22.22 ±11.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>18</td>
<td>63.33 ±23.62</td>
<td>-27.63 ±15.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>3</td>
<td>56.63 ±30.99</td>
<td>-32.90 ±4.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D2</td>
<td>GG</td>
<td>58</td>
<td>63.54 ±30.64</td>
<td>38.21 ±62.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>25</td>
<td>58.82 ±32.06</td>
<td>39.37 ±45.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>5</td>
<td>54.32 ±26.63</td>
<td>41.26 ±55.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D3</td>
<td>GG</td>
<td>47</td>
<td>66.52 ±22.63</td>
<td>51.73 ±49.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>33</td>
<td>53.71 ±25.14</td>
<td>95.47 ±119.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>6</td>
<td>39.27 ±15.61</td>
<td>107.47 ±55.8</td>
</tr>
<tr>
<td>rs11234027</td>
<td>South Asian</td>
<td>Placebo</td>
<td>TT</td>
<td>9</td>
<td>40.68 ±27.35</td>
<td>79.83 ±107.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>13</td>
<td>30.54 ±20.62</td>
<td>106.02 ±84.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>2</td>
<td>29.55 ±23.97</td>
<td>199.35 ±260.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D2</td>
<td>GG</td>
<td>11</td>
<td>25.54 ±20.38</td>
<td>268.79 ±185.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>7</td>
<td>32.84 ±18.70</td>
<td>133.14 ±151.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>2</td>
<td>36.65 ±39.81</td>
<td>374.45 ±545.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D3</td>
<td>GG</td>
<td>59</td>
<td>63.73 ±22.90</td>
<td>57.63 ±53.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>25</td>
<td>51.70 ±26.53</td>
<td>104.47 ±131.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>2</td>
<td>40.95 ±17.47</td>
<td>107.45 ±102.6</td>
</tr>
</tbody>
</table>

Key: Ethnic: ethnicity, Al: allele, Caul: Caucasian, **DHCR7**: 7-dehydrocholesterol reductase, blue shows p<0.005
7.3.4.2.1 rs12785878

Genotypes within the rs12785878 SNP showed a significant association with percentage change in 25OHD concentrations in the Caucasian cohort assigned the vitamin D₃ intervention (r²=0.066, p=0.017, n=). There was a positive association between the vitamin D risk allele (G) and percentage change in 25OHD concentrations (Figure 7-7).

Figure 7-7 Percentage change in 25OHD concentrations across genotypes of rs12785878, within each intervention group, split by ethnicity
Mean [± SEM]. Linear regression r² values are shown where significant *p<0.05
7.3.4.2.1 rs4944957

Genotypes within the rs4944957 SNP showed a significant association with percentage change in 25OHD concentrations in the Caucasian cohort assigned the vitamin D₃ intervention ($r^2=0.066$, $p=0.017$). There was a positive association between the vitamin D risk allele (A) and percentage change in 25OHD concentrations (Figure 7-8).

![Figure 7-8](image_url)

Figure 7-8 Percentage change in 25OHD concentrations across genotypes of rs4944957, within each intervention group, split by ethnicity
Mean [± SEM]. Linear regression $r^2$ values are shown where significant *$p<0.05$
7.3.4.2.2  rs11234027

Genotypes within the rs11234027 SNP showed a significant association with percentage change in 25OHD concentrations in the South Asian cohort assigned the placebo intervention ($r^2=0.527$, $p=0.011$), and the Caucasian cohort assigned the vitamin D$_3$ intervention ($r^2=0.060$, $p=0.023$). There was a positive association between the vitamin D risk allele (A) and percentage change in 25OHD concentrations in both cases (Figure 7-9).

![Figure 7-9 Percentage change in 25OHD concentrations across genotypes of rs11234027, within each intervention group split by ethnicity](image)

Mean [± SEM]. Linear regression $r^2$ values are shown where significant *$p<0.05$

7.3.5  CYP2R1 Gene

7.3.5.1  Discovery and Replication Findings

There was no association between genotype and 25OHD concentrations in either ethnic group, for either SNP within the CYP2R1 gene, in the discovery or the replication cohort (data not shown).
7.3.5.2 **Vitamin D RCT Cohort**

There were no significant associations between rs10741657 and percentage change in 25OHD concentrations in either ethnic group (data not shown). However, there was a significant association between percentage change in 25OHD concentrations and rs12794714 in the Caucasian vitamin D2 intervention group only (Table 7.8, data for South Asian cohort not shown as not significant).

### Table 7.8 Baseline and percentage change in 25OHD concentrations in individual SNPs within the CYP2R1 gene, and associations between SNP and percentage change in 25OHD concentrations in Caucasian women in the D2-D3 Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Treatment Group</th>
<th>Ethnic</th>
<th>Genotype</th>
<th>n</th>
<th>Baseline 25OHD (mmol/L) Mean ±SD</th>
<th>% Change in 25OHD Mean ±SD</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2R1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs12794714</td>
<td></td>
<td>Cauc</td>
<td>Placebo</td>
<td>15</td>
<td>62.88 ±20.44</td>
<td>-24.75 ±14.5</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>22</td>
<td>55.60 ±24.73</td>
<td>-23.05 ±13.3</td>
<td>-0.148</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>7</td>
<td>57.50 ±23.59</td>
<td>-32.67 ±8.7</td>
<td>0.336</td>
</tr>
<tr>
<td></td>
<td>Vitamin D2</td>
<td></td>
<td>GA</td>
<td>29</td>
<td>53.06 ±30.57</td>
<td>59.17 ±64.4</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>7</td>
<td>59.23 ±24.62</td>
<td>22.50 ±31.8</td>
<td>0.239</td>
</tr>
<tr>
<td>Vitamin D3</td>
<td></td>
<td></td>
<td>GA</td>
<td>50</td>
<td>67.11 ±30.96</td>
<td>29.76 ±53.9</td>
<td>0.025</td>
</tr>
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<td>AA</td>
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<td>59.23 ±24.62</td>
<td>22.50 ±31.8</td>
<td>0.025</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>28</td>
<td>57.79 ±21.80</td>
<td>98.34 ±126.1</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GA</td>
<td>41</td>
<td>63.94 ±24.90</td>
<td>53.14 ±52.8</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>AA</td>
<td>17</td>
<td>52.64 ±26.86</td>
<td>76.13 ±60.4</td>
<td>0.024</td>
</tr>
</tbody>
</table>

Key: Ethnic: ethnicity, Cauc: Caucasian, Al: alleles, CYP2R1: cytochrome P450, family 2, subfamily R, polypeptide 1. Blue shows p<0.005

7.3.5.2.1 **rs12794714**

Genotypes within the rs12794714 SNP showed a significant association with percentage change in 25OHD concentrations in the Caucasian cohort assigned the vitamin D2 intervention (r²=0.057, p=0.025) (Figure 7-10). There was a negative association between the vitamin D risk allele (A) and percentage change in 25OHD concentrations.
Figure 7-10 Percentage change in 25OHD concentrations across genotypes of rs12794714, within each intervention group split by ethnicity
Mean [± SEM]. Linear regression r² values are shown where significant *p<0.05

7.3.6 CYP24A1 Gene

7.3.6.1 Discovery and Replication Findings
There were no significant associations between rs6013897 genotype and 25OHD concentrations in the discovery and replication cohorts, in both South Asian and Caucasian cohorts (data not shown).

7.3.6.2 Vitamin D RCT Cohort
There were no significant associations between rs6013897 genotype and percentage change in 25OHD concentrations in any of the intervention groups within either ethnic group (data not shown).
7.3.7 **CYP27B1** Gene Results

7.3.7.1 **Discovery and Replication Findings**

There was a significant association between genotype and 25OHD concentrations within rs10877012, in the discovery cohort only (Table 7.9).

**Table 7.9** 25OHD concentrations of individual SNPs in the **CYP27B1** gene in South Asian and Caucasian women, and associations between SNP and 25OHD concentrations in both the discovery and replication cohort

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethnic</th>
<th>Al</th>
<th>25OHD (nmol/l)</th>
<th>Linear Regression</th>
<th>25OHD (nmol/l)</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>r² beta p-value</td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td><strong>CYP27B1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs10877012</td>
<td>South</td>
<td>GG</td>
<td>24 21.63 ±10.48</td>
<td>0.002 0.707</td>
<td>21 23.85 ±15.84</td>
<td>0.002 -0.71</td>
</tr>
<tr>
<td></td>
<td>Asian</td>
<td>GT</td>
<td>40 23.52 ±12.94</td>
<td>0.042</td>
<td>42 32.07 ±23.61</td>
<td>0.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>18 22.77 ±8.38</td>
<td></td>
<td>18 26.03 ±27.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cauc</td>
<td>GG</td>
<td>126 45.94 ±18.98</td>
<td>0.017 <strong>0.036</strong></td>
<td>111 58.19 ±25.91</td>
<td>0.000 0.747</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GT</td>
<td>99  49.36 ±20.36</td>
<td>0.132</td>
<td>97  63.38 ±27.33</td>
<td>0.021</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT</td>
<td>28  54.46 ±26.32</td>
<td></td>
<td>26  55.51 ±23.69</td>
<td></td>
</tr>
</tbody>
</table>

Key: Ethnic: ethnicity, Al: alleles, Cauc: Caucasian, CYP27B1: cytochrome P450, family 27, subfamily B, polypeptide 1, blue shows p<0.005

7.3.7.1.1 **rs10877012**

There was a significant positive correlation between rs10877012 genotypes and 25OHD concentrations in the Caucasian cohort, in the discovery cohort (r²=0.017, p=0.038), with the vitamin D risk allele (T) being associated with a higher 25OHD concentration, however there was no association in the replication cohort (Figure 7-11). In the South Asian groups there were no associations in either the discovery or replication cohort.
Figure 7-11 Scatterplot showing association between rs10877012 genotype and total 25OHD concentration in the discovery* and replication cohorts, in Caucasian women
Linear regression in discovery and replication cohorts: Caucasian (r^2=0.012* and r^2=0.000 respectively) women. Significant correlations indicated by asterisks, *p<0.05

7.3.7.2 Vitamin D RCT Cohort

There were no significant associations between genotype and percentage change in 25OHD concentrations for rs10877012, in any of the intervention groups within either ethnic group (data not shown).

7.3.8 VDR Gene

7.3.8.1 Discovery and Replication Findings

The only SNP to show an association between genotype and 25OHD concentration was rs7975232 (Table 7.10, data for all other SNPs not shown).
Table 7.10 25OHD concentrations of individual SNPs in the VDR gene in South Asian and Caucasian women, and associations between SNP and 25OHD concentrations in both the discovery and replication cohort

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethnic/Al</th>
<th>Discovery Cohort: D-FINES Study</th>
<th>Replication Cohort: D2-D3 Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>25OHD (nmol/l)</td>
<td>Linear Regression</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>VDR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs7975232</td>
<td>South Asian AA</td>
<td>26</td>
<td>18.08 ±6.63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AC</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>Caucasian AA</td>
<td>67</td>
<td>48.42 ±22.23</td>
</tr>
<tr>
<td></td>
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<td>AC</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CC</td>
<td>72</td>
</tr>
</tbody>
</table>

Key: Ethnic: ethnicity, Al: alleles, Cauc: Caucasian, VDR: vitamin D receptor, blue shows p<0.005

### 7.3.8.1.1 rs7975232

There was a significant positive correlation between rs7975232 genotype and 25OHD concentrations in the South Asian cohort in the discovery cohort (r²=0.062, p=0.026), with the vitamin D risk allele (C) being associated with a higher 25OHD concentrations, however, there was no association in the replication cohort (Figure 7-12). There were no associations within the Caucasian cohort.

![Figure 7-12 Scatterplots showing association between rs7975232 genotype and total 25OHD concentrations in the discovery and replication cohorts, in South Asian women](image-url)

Linear regression in discovery and replication cohorts: South Asian (r²=0.062* and r²=0.001 respectively). Significant correlations indicated by asterisks, *p<0.05
7.3.8.2 Vitamin D RCT Cohort

There were significant associations between genotype and percentage change in 25OHD concentrations for rs2228570 and rs731236 only (Table 7.11, data for the three other SNPs not shown as not significant).

Table 7.11 Baseline, week 12 and percentage change in 25OHD concentrations in individual SNPs within the VDR gene, and associations between SNP and percentage change in 25OHD concentrations in South Asian and Caucasian women in the D2-D3 Study

<table>
<thead>
<tr>
<th>SNP</th>
<th>Ethnic</th>
<th>Treatment Group</th>
<th>AI</th>
<th>Baseline 25OHD (nmol/L)</th>
<th>% Change in 25OHD</th>
<th>Linear Regression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>r²</td>
</tr>
<tr>
<td>VDR</td>
<td>South Asian</td>
<td>Placebo</td>
<td>TT</td>
<td>0</td>
<td>20.34 ±12.11</td>
<td>-15.82 ±38.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>5</td>
<td>26.82 ±10.86</td>
<td>-2.67 ±45.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>6</td>
<td>25.30 ±23.47</td>
<td>149.90 ±102.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D2</td>
<td>TT</td>
<td>1</td>
<td>25.7</td>
<td>80.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>8</td>
<td>39.61 ±22.77</td>
<td>81.07 ±110.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D3</td>
<td>TT</td>
<td>0</td>
<td>42.96 ±23.61</td>
<td>109.36 ±157.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>12</td>
<td>20.17 ±14.74</td>
<td>317.46 ±220.1</td>
</tr>
<tr>
<td></td>
<td>Caucasian</td>
<td>Placebo</td>
<td>TT</td>
<td>5</td>
<td>77.16 ±11.44</td>
<td>-26.48 ±17.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>19</td>
<td>54.29 ±20.20</td>
<td>-22.89 ±12.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>CC</td>
<td>20</td>
<td>57.58 ±25.70</td>
<td>-27.0 ±14.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vitamin D2</td>
<td>TT</td>
<td>15</td>
<td>52.73 ±21.03</td>
<td>39.45 ±44.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>TC</td>
<td>34</td>
<td>57.35 ±30.07</td>
<td>49.62 ±166.1</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>37</td>
<td>67.80 ±31.59</td>
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<td>107.88 ±186.9</td>
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<td>TC</td>
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<td>101.66 ±60.7</td>
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<td>CC</td>
<td>17</td>
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</table>

Key: VDR: vitamin D receptor, blue shows p<0.005, red shows p<0.008
7.3.8.2.1 rs2228570

Genotypes within the rs2228570 SNP showed a significant association with percentage change in 25OHD concentrations in the South Asian cohort assigned the vitamin D₃ intervention (r²=0.22, p=0.043). There was a positive association between the vitamin D risk allele (C) and percentage change in 25OHD concentrations (Figure 7-13).

![Figure 7-13 Percentage change in 25OHD concentrations across genotypes of rs2228570, within each intervention group split by ethnicity](image)

Mean [± SEM]. Linear regression r² values are shown where significant *p<0.05

7.3.8.2.2 rs731236

Genotypes within the rs731236 SNP showed a significant association with percentage change in 25OHD concentrations in both the South Asian placebo cohort (r²=0.595, p=0.005) and the Caucasian placebo cohort (r²=0.107, p=0.030). There was a positive association between the vitamin D risk allele (A) and percentage change in 25OHD concentrations in both cases (Figure 7-14).
7.3.9 Genotype and Vitamin D Binding Protein

7.3.9.1 Participant Characteristics

Out of a possible total of 90 South Asian and 234 Caucasian women recruited as part of the D2-D3 Study, 16 South Asian and 53 Caucasian participants were included in these analyses. These participants consented to genetic analyses, successfully completed the 12-week intervention and had plasma concentrations of VDBP measured, in addition to 25OHD concentrations, and all other measurements taken as part of the D2-D3 study.

7.3.9.2 Results

There was a negative association between the presence of risk alleles and VDBP concentrations for rs4588 within the Caucasian participants ($r=-0.427, p=0.001$), and rs7041 within both the South Asian and Caucasian participants ($r=-0.689, p=0.002$ and $r=-0.675, p<0.001$ respectively). In all cases the minor allele related to reduced VDBP concentrations (Figure 7-15 and Figure 7-16).

Figure 7-14 Percentage change in 25OHD concentration across genotypes of rs731236, within each intervention group split by ethnicity
Mean [± SEM]. Linear regression $r^2$ values are shown where significant *$p<0.05$, **$p<0.008$. 
7.3.10 Genotype and Bone Density

7.3.10.1 Participant Characteristics

Out of a possible total of 90 South Asian and 234 Caucasian women recruited as part of the D2-D3 Main Study, 81 South Asian and 234 Caucasian women gave consent to be part of the D2-D3 Genetic Study and had a pQCT scan of the radius completed. The South Asian cohort had a mean 25OHD concentration of 28.60 ±22.86 nmol/L, mean age of 37.65 ±10.35 years and a mean BMI of 25.44
±4.39 kg/m². The Caucasian cohort had a mean 25OHD concentration of 60.05 ±26.33 nmol/L, mean age of 45.58 ±12.31 years and a mean BMI of 23.61 ±3.44 kg/m².

**7.3.10.2 Results**

In partial correlation analyses, controlling for baseline age, BMI, parathyroid hormone, corrected calcium, and 25OHD concentrations, there was a significant positive correlation between 25OHD lowering/risk alleles within rs4588 genotype and vBMD (r=0.160, p=0.016, Figure 7-17).

![Figure 7-17 Scatterplots showing association between volumetric bone mineral density (mg/cm³) and rs4588 genotype in South Asian and Caucasian women](image)

*Key: Genotype 0=CC, 1=CA, 2=AA. Caucasian cohort: r=0.160, p=0.016*

**7.4 DISCUSSION**

This study aimed to investigate whether SNPs, within genes with functions in the metabolism of vitamin D, were associated with 25OHD concentrations, VDBP concentrations and vBMD, in both South Asian and Caucasian women. It also aimed to investigate whether those SNPs were also association with response to 15 µg/d (600 IU/d) vitamin D₂ vs. vitamin D₃, in both South Asian and Caucasian women.
This study is the first to have examined the association between common polymorphisms and percentage change in 25OHD concentrations in response to 15μg/d vitamin D₂, 15μg/d vitamin D₃, or placebo, delivered via fortified foods, in both South Asian and Caucasian healthy female cohorts. If the UK were to implement a population-wide vitamin D fortification strategy or policy to improve vitamin D status it would be important to know whether individuals may respond differently to supplementation as a result of a genetic predisposition.

### 7.4.1 Summary of all Genes

Across all six genes, there were no significant associations with 25OHD concentrations that were identified in both the discovery and the replication cohort, however some independent associations were identified in the *DHCR7* gene, *CYP27B1* gene and *VDR* gene. There were also associations between SNPs within the *GC* gene, *DHCR7* gene, *CYP2R1* gene, and *VDR* gene and percentage change in 25OHD concentrations, which varied between ethnic group and intervention group, and warrant further research in a larger sample size with greater control for baseline 25OHD concentrations.

### 7.4.2 GC Gene

*GC*, also known as the group-specific component, encodes for the VDBP and is a major transporter of vitamin D metabolites; transporting vitamin D to the liver, 25OHD to the kidney and other organs, and 1,25(OH)₂D to target organs (Daiger *et al.* 1975). It has been suggested that the mechanism of action by which genotypes could be associated with 25OHD concentrations is due to the changes in the VDBP creating different affinities for 25OHD and 1,25(OH)₂D (Constans *et al.* 1980), but also varying plasma concentrations of the VDBP (Lauridsen *et al.* 2005).

#### 7.4.2.1 Association with 25OHD Concentrations

The two SNPs within the GC gene (rs4588 and rs7041) did not show any associations with 25OHD concentrations in either the discovery or the replication cohorts. These findings conflict with several previous findings, from both candidate gene studies and GWAS. In such candidate gene studies (Engelman *et al.* 2008; Fu *et al.* 2008; Kurylowicz *et al.* 2006; Sinotte *et al.* 2009; Perna *et al.* 2013) the minor allele (A) of rs4588 has been consistently associated with lower 25OHD concentrations. A perfect proxy for rs4588 is rs2282679 (Moy *et al.* 2014), which has also been shown to be significantly associated with 25OHD concentrations in candidate gene studies (Ahn *et al.* 2009) and GWAS (Ahn *et al.* 2010; Wang *et al.* 2010). In the candidate gene studies which have shown an
association between 25OHD concentrations and rs7041 genotypes (Ahn et al. 2009; Engelman et al. 2008; Sinotte et al. 2009), and the GWAS (Ahn et al. 2010; Wang et al. 2010), again the minor allele (T) has been repeatedly associated with lower 25OHD concentrations. Specifically within South Asian populations, both rs2292679 (perfect proxy for rs4588) and rs7041 have been shown to be significantly associated with 25OHD concentrations (Elkum et al. 2014), which conflicts with the findings of the current study. It is likely that the D2-D3 Study was underpowered, as these previous studies have all had participant numbers >1,549.

7.4.2.2 Association with Percentage Change

When investigating associations between genotype and percentage change in 25OHD concentrations, the two SNPs within the GC gene showed a significant association with percentage change in the South Asian placebo group only, and no association with change in 25OHD for either ethnic groups on either vitamin D₂ or vitamin D₃ intervention. This study found that both rs4588 and rs7041 of the GC gene were associated with percentage change in 25OHD concentrations in the South Asian placebo interventions only, but not in response to either supplementation intervention. The data for both SNPs showed that those with one risk allele did not show a decrease in 25OHD concentrations over the winter period, as was seen in those with no risk alleles. However, those with one risk allele had much lower 25OHD concentrations at baseline and it is therefore possible that this association was actually due to a protective mechanism against low 25OHD concentrations becoming even lower, as opposed to a direct effect of the SNP genotype on change in 25OHD. A previous study has also shown that those with two risk alleles for rs4588 show a greater increase in 25OHD concentrations after one year of supplementation with 150 μg/d (6000 IU/d) vitamin D₃, than those with no risk alleles, but also had lower 25OHD concentrations at baseline, and so again whether this finding is due to an adaptive response of the genotype, or just an adaptive response to the lower vitamin D concentrations it is not clear (Fu et al. 2009). However, in a study giving 10 μg/d (400 IU/d) of vitamin D₂ or vitamin D₃ for three months the opposing association was found following the vitamin D₃ intervention, but not the vitamin D₂ intervention (Nimitphong et al. 2013). From the current and previous studies no clear conclusions can be drawn.

7.4.3 DHCR7 Gene

7.4.3.1 Association with 25OHD Concentrations

In the DHCR7 gene, both rs12785878 and rs4944957 showed significant negative associations with 25OHD concentrations in the South Asian group within the discovery cohort but not in the replication
cohort, whereas in the Caucasian group there was no significance in the discovery cohort, but there was a significant association in the replication cohort. These associations were significant when a p-value of <0.05 was applied, but not when more stringent testing was applied (p<0.008). The only association significant at this level, across all six genes, was for rs11234027 within the DHCR7 gene in the South Asian group of the discovery cohort, where a significant negative association was found between 25OHD concentrations and genotype. This has previously been shown in GWAS for rs3829251, which has been shown to be a perfect proxy for rs11234027 (Ahn et al. 2010). However, this finding was not found in the replication cohort.

### 7.4.3.2 Association with Percentage Change

When looking at associations between genotype and percentage change in 25OHD concentrations, all three SNPs in the DHCR7 gene showed an association with percentage change in 25OHD in the Caucasian vitamin D3 group, with the alleles that have previously been associated with lower 25OHD concentrations showing a greater percentage increase in 25OHD in response to vitamin D3 intervention. Across all three SNPs, although those with no minor alleles showed a 52-58% increase, and those with two minor alleles showed a 107% increase, at the end of the 12-week study those with two minor alleles still had a lower 25OHD concentration than those with no risk alleles. Although it cannot be elucidated from these analyses whether the effect on change in 25OHD concentrations is as a result of the genotype or baseline 25OHD concentrations themselves, it is curious that this finding was only present in response to the vitamin D3 intervention and within the Caucasian group. The DHCR7 gene encodes 7-dehydrocholesterol reductase, which catalyses the conversion of 7-dehydrocholesterol to cholesterol, removing it from the substrate pathway of vitamin D3. It is therefore biologically plausible that genetic polymorphisms within the DHCR7 gene could influence response to supplementation, specifically vitamin D3 as shown in this study.

### 7.4.4 CYP2R1 Gene

#### 7.4.4.1 Association with 25OHD Concentrations

In both the discovery and replication cohorts, none of the SNPs within the CYP2R1 gene (rs10741657 and rs12794714) showed any association with 25OHD concentrations. This conflicts with findings from candidate gene studies (Ramos-Lopez et al. 2007) and GWAS (Ahn et al. 2010; Wang et al. 2010). Although, Nissen and colleagues (2015) also found no association between 25OHD concentrations and rs10741657 in a cohort of 92 Danish participants, however, study numbers
within the Danish cohort (Nissen et al. 2015) and this study cohort were much lower than the studies that have found associations.

7.4.4.2 Association with Percentage Change

When investigating associations between genotype and percentage change in 25OHD concentrations, the only significant association found in the current study was for rs12794714 within the Caucasian vitamin D$_2$ intervention group, where the minor allele was associated with a lower percentage change in 25OHD. This is despite not having the highest baseline 25OHD concentration, and so is not a finding clearly influenced by baseline 25OHD.

_CYP2R1_ encodes for 25-hydroxylase, which is an enzyme that catalyses the hepatic hydroxylation of vitamin D to 25OHD (Wang et al. 2010). The finding within this SNP could suggest that response to vitamin D$_2$ may be influenced by genotype within rs12794714, which may be a result of changes in the capacity of CYP2R1 to catalyse the hydroxylation of vitamin D$_2$ to 25OHD$_2$. No previous known study has examined associations between this SNP and change in 25OHD concentrations, however a study has looked at rs10741657 (the other SNP included in this analysis) in this context and found that the polymorphism is able to predict UVB-induced 25OHD concentrations, with those with the risk allele (the major allele in this case) associated with a lower total increase in 25OHD concentrations (Nissen et al. 2015). We did not find any association between rs10741657 and percentage change in 25OHD, in response to either vitamin D$_2$ or vitamin D$_3$.

7.4.5 CYP24A1 Gene

7.4.5.1 Association with 25OHD Concentration

In both the discovery and replication cohorts, rs6013897 within the _CYP24A1_ gene did not show any association with 25OHD concentration. These findings conflict with previous GWAS findings (Ahn et al. 2010; Wang et al. 2010), however, in support of our findings, Kuhn and colleagues (2013) also found no associations between rs6013897 and 25OHD concentrations, despite being a well powered study (n=52,988).

7.4.5.2 Association with Percentage Change

There were also no significant associations between this SNP within the _CYP24A1_ gene and percentage change in 25OHD concentrations in response to the D2-D3 intervention groups. No study to date has studied associations between this SNP and change in 25OHD concentrations, and therefore it is difficult to draw any firm conclusions based on the findings of the current study alone.
7.4.6 CYP27B1 Gene

7.4.6.1 Association with 25OHD Concentration

In the discovery cohort there was a significant positive association between rs10877012 of the CYP27B1 gene and 25OHD concentrations (Caucasians only), however this finding was not found in the replication cohort, nor was it present in the South Asian cohorts. These findings contrast with previous findings from candidate gene studies (Berry et al. 2012), however, Bu and colleagues (2010) have previously found no association between this SNP and 25OHD concentrations and GWAS have also not identified any SNPs within the CYP27B1 gene (Wang et al. 2010; Ahn et al. 2010).

7.4.6.2 Association with Percentage Change

There were also no significant associations between this SNP within the CYP27B1 gene and percentage change in 25OHD in response to the D2-D3 intervention groups. No study to date has examined associations between this SNP and change in 25OHD concentrations, and therefore definitive conclusions cannot be drawn.

7.4.7 VDR Gene

7.4.7.1 Association with 25OHD Concentrations

In the discovery cohort there was a significant positive association between rs7975232 of the VDR gene and 25OHD concentrations, however this finding was not found in the replication cohort, nor was it present in the South Asian cohorts. There were also no associations with 25OHD concentrations for any of the four other SNPs. These findings were consistent with previous findings from candidate gene studies in Caucasian subjects (n=156) (Bu et al. 2010), which found no association between any of the five SNPs and 25OHD concentrations.

7.4.7.2 Association with Percentage Change

There was an association between rs2228570 and percentage change in 25OHD in the South Asian vitamin D3 cohort only, and rs731236 showed a significant association in both the South Asian placebo cohort and the Caucasian placebo cohort. In both the South Asian cohorts, a positive association was shown with a greater percentage change in 25OHD concentrations shown in those with risk alleles. However, in the Caucasian placebo cohort the association was negative. No study to date has looked at associations between these SNPs and change in 25OHD concentrations, limiting any comparisons which can be made or conclusions which can be drawn from this. It would be particularly interesting to look at associations between SNPs within the VDR and 1,25(OH)2D.
concentrations, as opposed to 25OHD concentrations, as it is the active form of vitamin D (1,25(OH)$_2$D) that binds to the VDR.

7.4.8 Vitamin D Binding Protein

In this analysis, both of the SNPs within the GC gene showed an association with baseline VDBP concentrations in the Caucasian cohort, with rs7041 also showing an association within the South Asian cohort. For rs7041 this study showed lower VDBP concentrations with increasing copies of the minor allele (T), with the South Asian and Caucasian cohorts showing a 76% and 61%, respectively, lower VDBP concentration in those with two copies of the minor allele (TT) compared to those with no copies of the minor allele (GG). This finding is consistent with previous findings by Moy et al. (2014), who showed a 57% lower VDBP concentration in their GWAS in healthy 50-69 year old males from Finland. The D2-D3 study dataset also showed that rs4588 had a significant association with VDBP concentrations within the Caucasian cohort, with those with two copies of the minor allele (AA) having a 49% lower VDBP concentration than those with no copies of the minor allele (CC). This was not replicated in the South Asian cohort, although there were no South Asian participants with the AA allele in this dataset, limiting the ability to investigate this association. The same direction of change was shown, with those with one copy of the minor allele (CA) having a 13% lower VDBP concentration, although this was not a significant difference.

These data, along with previous studies (Powe et al. 2013) suggest that those with the alleles associated with lower 25OHD concentrations (from previous GWAS) also have lower VDBP concentrations. Powe and colleagues (2013) suggested that the parallel associations between 25OHD and VDBP concentrations is likely to result in a similar level of bioavailable vitamin D to those with higher 25OHD concentrations (and higher VDBP concentrations).

7.4.9 Volumetric Bone Mineral Density

Within the Caucasian cohort there was an association between rs4588 of the GC gene and vBMD, with bone density increasing with the presence of alleles associated with lower 25OHD concentrations (Wang et al. 2010; Ahn et al. 2010). The GC gene encodes the VDBP and so one theory for this finding is that, although 25OHD concentrations are associated with being lower in those with vitamin D lowering alleles of rs4588, VDBP concentrations are also lower and therefore the overall affect is more free and bioavailable 25OHD (Powe et al. 2013).
7.4.10 Ethnic Differences

There were differences in gene allele frequencies and MAF between the Caucasian and South Asian cohorts for SNPs within the DHCR7 gene, but the allele frequencies for SNPs in other genes did not vary. Despite similarities in allele frequencies, there were differences between the ethnic groups in terms of SNPs that were associated with baseline 25OHD concentrations and percentage in 25OHD concentrations in response to supplementation. This supports previous studies that have shown ethnic differences in SNP associations with 25OHD concentrations (Batai et al. 2014; Elkum et al. 2014; Suaini et al. 2014), but goes further to suggest that response to supplementation may possibly also be influenced by ethnicity-specific genetic variants.

7.4.11 Strengths

One of the strengths of this study is that it was conducted in healthy populations, and thus the impact of disease is limited. The collection of data from both Caucasian and South Asian women is also a strength, in particular having both the baseline and randomised-controlled trial data.

7.4.12 Limitations

One of the main limitations of this study is participant numbers, particularly within the South Asian cohort. Across all three cohorts, there were no consistent findings within the South Asian cohort, and this is possibly due to a lack of power in this ethnic group. Not only were the whole study numbers lower in the South Asian cohort, but there were also several cases in which the number of participants with the minor homozygous allele were <10. The only way to overcome this limitation would be to screen by genotype at recruitment, which would have substantial implications for the timescale and costs of the study.

Another limitation of these analyses are the potential confounding effects of baseline 25OHD concentrations on associations found between SNPs and percentage change in 25OHD concentrations.

Across all six genes, and 14 SNPs, there were no significant associations with 25OHD concentrations that were identified in both the discovery and the replication cohort. One of the possible explanations for this could be due to the time of year in which the samples were collected. The
discovery/D-FINES Study collected samples at each season and where data were missing from all other seasons, summer 25OHD concentrations were used (14.6%), whereas in the replication/D2-D3 Genetic Study all samples were collected during the winter months. Studies have shown that the relation of GC gene SNPs rs4588 and rs7041 with 25OHD concentrations were more apparent during the summer or autumn months than the winter months (Sinotte et al. 2009; Perna et al. 2013; Engelman et al. 2013), and this is also supported by a study that has shown no association between GC SNPs and 25OHD concentrations during the winter months (Gozdzik et al. 2011). During winter, the vitamin D sequestered and stored during the summer months is used, and therefore the genetic variants in genes involved in the metabolism of vitamin D may not predict 25OHD concentration.

Another limitation of this dataset is the different laboratory methods used to determine 25OHD concentrations for the D2-D3 Study and D-FINES study, and also in the studies that were being compared to throughout. Although both sets of samples were analysed by the same team (Dr Jackie Berry, University of Manchester), the D-FINES Study was completed in 2008 whereas the D2-D3 Study was completed in 2013, during which time the labs method for analysing 25OHD concentrations had changed from a manual enzyme immunoassay kit to LC-MS/MS.

It is possible that publication bias is an issue in this field, as studies that did not find associations between genotypes and 25OHD concentrations may be under reported.

7.4.13 Further Work

Analysing the data using a statistical package such as PLINK or R, specifically designed for genetic analyses, may be required for any publication of these data. This is a future plan of analysis for the dataset.

Within the D2-D3 Study, a further 16 SNPs have also been genotyped, but were not presented here. However, as our sample size is limited, combining these data with a larger dataset, or a consortium, could help to improve the quality of the analyses and findings. The need to elucidate the ethnic variation in SNPs and the association with 25OHD concentrations and response to supplementation would also be improved with a larger sample size, and a broader inclusion of ethnic groups. Greater control or consideration for baseline 25OHD concentrations would also improve the ability to draw firm conclusions from any findings comparing genotype with change in 25OHD concentrations.
This study, along with several other studies, examined the associations between individual SNPs and 25OHD concentration or percentage change in 25OHD concentrations in response to vitamin D supplementation. However, it may not be a single SNP that is the true disease causing variants, but it may be a combination of genetic variants that is important, or that the identified genetic variant is in linkage disequilibrium with another genetic variant that is the true variant, making the identified SNP a useful marker of risk rather than the true cause. Developing genetic risk scores (GRS) based on a combination of SNPs is one way of investigating this.

It would also be interesting to examine associations between SNPs, particularly within the VDR, with 1,25(OH)₂D concentrations, as well as associations with 25OHD₂ and 25OHD₃ specifically.

Although there are no plans for this at present, possible future mechanisms that may help to identify these remaining determinants include next generation DNA sequencing and mendelian randomisation. Next generation sequencing includes rare genetic variants with minor allele frequencies <1%. Despite being rare, these variants may have a large effect on 25OHD concentrations and, if identified, may help to build a complete picture on the heritability and genetic determinants of vitamin D status and response to supplementation.

7.4.14 Conclusion

This study does not show conclusive evidence of associations between genetic variants and 25OHD concentrations, as there were no consistent results between the discovery and replication cohorts. However, the study numbers are likely to be the limiting factor in this, as there is a host of prior evidence showing associations between the selected SNPs and 25OHD concentrations.

Although associations between percentage change in 25OHD concentrations and SNPs within the GC gene and DHCR7 gene were shown, a possible explanation for the differences seen could be a result of differences in baseline 25OHD concentrations, as opposed to the effect of the genetic variant on response to vitamin D₂ or D₃ per se.
Chapter 8 - GENERAL DISCUSSION
8.1 Summary of Thesis Results and Original Contributions

Chapter 1 highlighted the problem of vitamin D deficiency and insufficiency in the UK, and how season and region (latitude) are known to affect this, as well as the issue that South Asian populations are known to have lower vitamin D status. It also identified the limited availability of natural sources of vitamin D in the UK, both dietary and sunlight; and concomitantly how supplementation and food fortification are likely to be key strategies for individuals to achieve the UK dietary recommendation of 10μg/d that have been proposed by SACN (SACN 2015). Previous literature on the efficacy of the two forms of vitamin D available for supplementation or food fortification; vitamin D$_2$ and vitamin D$_3$, showed inconsistencies and lacked study power to show conclusive findings.

This Thesis aimed to address key gaps in the literature. First, the efficacy of vitamin D$_2$ compared with vitamin D$_3$ in raising vitamin D status, delivered via fortified foods, in both South Asian and Caucasian women. Second, the effect of vitamin D$_2$ compared with vitamin D$_3$ on health outcomes; namely, bone health and cardiovascular health parameters. Third, the effect of vitamin D$_2$ compared with vitamin D$_3$ on gene expression and subsequent metabolic and cellular pathway activity. Fourth, the effect of genetic variants/polymorphisms on the change in vitamin D status seen in response to vitamin D$_2$ compared with vitamin D$_3$.

8.1.1 The D2-D3 Study

The D2-D3 Study is a randomised placebo controlled trial, primarily designed to compare the efficacy of 15 μg/d vitamin D$_2$ vs 15 μg/d vitamin D$_3$, delivered via fortified foods, at raising serum 25OHD concentration. Caucasian ($n$ 245) and South Asian ($n$ 90) women were recruited onto the 12-week intervention, and were given specially prepared intervention foods (orange juice (fluid vehicle) and a biscuit (solid vehicle)) to consume daily alongside their normal diet.

Primarily, the D2-D3 Study, presented in Chapter 3, showed that vitamin D$_3$ was 1.8 to 2.4 times more effective than vitamin D$_2$ at raising total serum 25OHD concentrations in both Caucasian women, who had ‘sufficient’ vitamin D concentrations at baseline, and South Asian women, who had ‘deficient’/‘insufficient’ vitamin D concentrations at baseline. Previous studies have shown varied results, and although a meta-analysis of all studies comparing the efficacy of vitamin D$_2$ and vitamin D$_3$ showed that vitamin D$_3$ was the more effective form, this finding was not the case when only studies that gave daily doses were considered (Tripkovic et al. 2012). The D2-D3 study is the largest randomised-controlled trial to compare the efficacy of vitamin D$_2$ and vitamin D$_3$ at raising 25OHD concentrations, and provides conclusive evidence that vitamin D$_3$ is superior under these experimental
It is important to note, however, that both vitamin D$_2$ and D$_3$ led to a significant increase in serum 25OHD concentration, but that the increase in the 12-week study period was greater in response to the vitamin D$_3$ intervention.

The vitamin D$_2$ intervention led to an increase in 25OHD$_2$ and a decrease in 25OHD$_3$ concentrations, whereas the vitamin D$_3$ intervention led to an increase in 25OHD$_3$ and no change in 25OHD$_2$ concentrations. This decrease in 25OHD$_3$ concentrations within those on the vitamin D$_2$ intervention explains to a large extent why total 25OHD concentrations do not increase as much in response to vitamin D$_2$ as they do in response to vitamin D$_3$.

Additionally, this study showed that four weeks after the 12-week intervention had ended, total 25OHD concentrations in those who had been on the vitamin D$_2$ intervention had returned to baseline, whereas the total 25OHD concentrations in those who had been on the vitamin D$_3$ intervention continue to remain higher than at baseline. This suggests that the degradation of 25OHD$_2$ is more rapid than 25OHD$_3$ concentrations, and therefore vitamin D$_3$ is not only more effective than vitamin D$_2$ at raising 25OHD concentrations, but also at maintaining concentrations within this four week time period.

This study also showed that 15 μg/d of vitamin D$_3$ over 12-weeks was a sufficient dose to raise winter-time 25OHD concentrations above the ‘deficiency’ threshold of <25 nmol/L in all of the South Asian and Caucasian women, and above the ‘insufficiency’ threshold of 25-50 nmol/L in all of the Caucasian women, whereas 15 μg/d of vitamin D$_2$ resulted in a small proportion of the South Asian women (11%) still having ‘deficient’ 25OHD concentrations.

The two forms of vitamin D were delivered via two different foods; orange juice or biscuit, to determine which vehicle was more effective for delivery of vitamin D. This study showed that both vehicles were equally effective at raising 25OHD concentrations, and therefore the composition of the orange juice and the biscuit, in particular the higher fat content of the biscuit, did not affect the availability of either vitamin D$_2$ or vitamin D$_3$.

8.1.2 Bone Health

Chapter 4 highlighted that South Asian women, who had significantly lower 25OHD concentrations than the Caucasian women, also had significantly higher parathyroid hormone (PTH) concentrations at
baseline. This could have important implications for the bone health of the South Asian women, and the measurement of bone turnover markers is an area for further future research.

The study also showed that the South Asian women had smaller, yet more dense bones at the radius, compared with Caucasian women. However, this did not translate into stronger bones as the strength-strain index was significantly lower for the South Asian women. Although these findings at the radius between the ethnic groups is not an original finding, as it has previously been shown by Darling and colleagues (2013b), it makes a significant contribution to the limited data on volumetric bone mineral density in ethnic groups.

Unlike previous studies, the current study did not show any association between 25OHD concentrations and radial pQCT measures, which is most likely due to the recruitment criteria for the D2-D3 Study. Participants were not recruited if they had been on vitamin D supplements, or had been on a holiday prior to the study, and therefore the range of baseline 25OHD concentrations seen within the D2-D3 Study was narrower than many other studies.

In response to the D2-D3 randomised-controlled trial, this study showed that vitamin D₃, but not vitamin D₂, had beneficial effects on PTH concentrations. In the vitamin D₃ groups, PTH decreased in the South Asian women (p<0.001), who had higher baseline concentrations, and were maintained in the Caucasian women, who had lower, healthy PTH concentrations at baseline. Vitamin D₂ did not have this effect.

8.1.3 Cardiovascular Health

Chapter 5 showed significant differences between South Asian and Caucasian women in terms of body size and shape that would suggest that the South Asian women were at an increased risk of cardiovascular disease (CVD). However, blood pressure, total cholesterol and LDL cholesterol concentrations were significantly lower in the South Asian women, which was a most interesting finding and would suggest otherwise. A possible explanation for this could be the age of the participants; the South Asian women were an average significantly younger than the Caucasian women.

In this study, we showed no association between 25OHD concentrations and blood lipid concentrations. Similarly to the lack of association between 25OHD concentrations and radial pQCT measures, this could be due to the recruitment criteria for the D2-D3 Study. Not only were
participants excluded if they had been on vitamin D supplements or had excess exposure to UVB rays (holiday or sunbed use), but they were also excluded if they were on medications for high cholesterol.

In response to the D2-D3 intervention, this study did not show any clear, consistent, beneficial changes in blood lipid concentrations in response to either vitamin D2 or vitamin D3. This suggests no beneficial role for either vitamin D2 or vitamin D3 in the prevention of CVD. There were slight increases in triglyceride concentrations in response to vitamin D, which warrants further research. However it is important to note that the participants within this study had healthy blood lipid concentrations, and so findings cannot be applied to those with high blood lipid concentrations or a greater risk of CVD.

8.1.4 Gene Expression in Response to Vitamin D2 and D3

In Chapter 6, gene expression in response to vitamin D2 and vitamin D3 supplementation, delivered via fortified foods, was investigated. This is the first study to have examined gene expression in response to vitamin D2 and vitamin D3, and the largest study to have examined gene expression in response to vitamin D.

In response to vitamin D, with the D2 treated and D3 treated participants combined, 1,241 genes were shown to be differentially expressed following 12-weeks of vitamin D. This number of genes is far larger than any previous studies have identified, with >1000 novel genes shown to be differentially expressed following the vitamin intervention. This study also showed a greater number of genes to be down-regulated in response to the intervention, whereas previous studies have shown that more genes are up-regulated.

Change in gene expression within the vitamin D2 and vitamin D3 treated participants independently, showed that the top pathways in which genes are differentially expressed differ between those treated with vitamin D2 to those treated with vitamin D3. Whether one form produces more beneficial changes in gene expression than the other is yet to be fully elucidated from the data.

Further analysis and interpretation of results are underway to understand whether the responses seen to both forms are equally beneficial or not. The dataset available also gives us the opportunity to explore ethnic variations, although this is currently lacking power and funding is being sought to increase sample size.
8.1.5 Genetic Variants and Response to Vitamin D2 and D3

In this chapter, associations between individual SNPs and baseline 25OHD concentrations showed inconsistent results across the discovery and replication cohorts, and therefore no consistent association with 25OHD concentrations. This was the case for all of the SNPs in the analyses. As these SNPs were selected for analysis based on the fact that they had showed prior associations with 25OHD concentrations, it was not expected that such inconsistent results would be shown. Study numbers are a limitation to this dataset and the data may be of better use if included in a consortium of data such as the SUNLIGHT consortium (Wang et al. 2010).

In this chapter, associations between SNP variants and response (percentage change in 25OHD) to placebo, vitamin D2 and vitamin D3 were also examined in order to identify whether SNPs (that have previously been associated with 25OHD concentrations in large GWAS) explain why some individuals respond well to supplementation and others do not, as speculated by Wang and colleagues (2010). This study found that both rs4588 and rs7041 of the GC gene were associated with percentage change in 25OHD in the South Asian placebo interventions only, but not in response to either D2 or D3 supplementation. Also, three SNPs within the DHCR7 gene showed significant associations with percentage change in 25OHD in the Caucasian vitamin D3 intervention group. However, it is possible that the association shown were a result of differences in baseline 25OHD concentrations as opposed to the genetic variant per se. Further research into whether genetic variants affect response to either vitamin D2 or vitamin D3 supplementation is needed in larger sample sizes, controlling for baseline 25OHD concentrations, to elucidate associations between genotypes and response to supplementation.

8.2 Wider Implications of the Work

The implications of this work are not just relevant to the academic community, but also health professionals, policy makers, industry members and individuals in the general public.

8.2.1 Researchers

There has been ongoing controversy amongst the academic and research communities as to whether vitamin D2 and vitamin D3 are equally effective at raising 25OHD concentrations, and the findings from the D2-D3 study provide conclusive evidence to support the superiority of vitamin D3 over vitamin D2 in this respect. Continual use of vitamin D2 in supplementation research trials may only serve to confound the understanding of optimal vitamin D dosing recommendations, and interpretation of any
vitamin D supplementation trials must consider the form used. Research attention should now be focused on determining the further mechanisms explaining why vitamin D$_3$ is more effective.

This study also highlighted the importance of presenting not only total 25OHD concentrations, but also 25OHD$_2$ and 25OHD$_3$ concentrations. Without the measurement of these metabolites it would not have been possible to detect that the vitamin D$_2$ led to a decline in 25OHD$_3$, in turn affecting total 25OHD concentrations.

The form of vitamin D given, and the concentrations of 25OHD metabolites should be considered when associating vitamin D and/or vitamin D status to health, given that this study also showed that the changes in gene expression, and therefore metabolic and cellular changes, seen in response to vitamin D$_2$ and vitamin D$_3$ fortified foods differed.

**8.2.2 Health Professionals**

The implications of this work, as the largest RCT conducted to date, and showing conclusively that vitamin D$_3$ is more effective than vitamin D$_2$ at raising total 25OHD concentrations and achieving or maintaining healthy PTH concentrations, are important. In the clinical setting vitamin D$_3$ may be preferential in the treatment of vitamin D deficiency. It is, however, important to note that vitamin D$_2$ did increase 25OHD concentrations, but to a lesser extent than vitamin D$_3$.

This study also showed that, over a period of 12 weeks, 15 µg/d of vitamin D$_3$, but not vitamin D$_2$, was sufficient to raise 25OHD concentrations of all of the Caucasian and South Asian women above the deficient threshold of 25 nmol/L. When treating patients identified as deficient, a higher dose of vitamin D$_3$ would be required if targets were to achieve >25 nmol/L within a quicker time frame than 12 weeks. The recommendation for vitamin D of 10 µg/d, which has been proposed by SACN (2015) for the whole UK population, is suggested as a daily recommendation for maintaining 25OHD concentrations throughout the year. However, in the case of deficiencies, a short-term higher dose may be beneficial for achieving >25 nmol/L, followed by 10 µg/d to maintain concentrations.

**8.2.2.1 Supplementation of Vitamin D**

One possible strategy for improving vitamin D status is vitamin D supplementation. Since 1991, the UK has recommended supplementation of 10 µg/d for ‘at-risk’ groups, such as South Asian women. This study raises serious concerns about the implementation of this policy, as there were still a number of South Asian women, who would be considered at-risk and who had 25OHD concentrations <25 nmol/L,
available for recruitment onto the study, although several women were excluded at the recruitment stages due to vitamin D supplementation prescribed by their GP. This suggests that at the time of recruitment (2011-2012) implementation of public policy, to supplement with 10 μg/d for those at-risk of vitamin D deficiency, had not reached all of the community who are indeed at-risk. Whether this is due to insufficient screening for deficiencies by health professionals, or barriers to taking vitamin D supplements within this population group, is not clear. In 2012 the Chief Medical Officer wrote to General Practitioners and Practice Nurses to increase awareness of the issue of vitamin D deficiency, and highlighted the recommendations for those at risk (Davies et al. 2012).

8.2.3 Public Health/Policy Makers
At present, the guidelines and recommendations for vitamin D requirements worldwide do not specify a form of vitamin D. However, as this study demonstrates clear differences in the efficiency of the two forms, at raising 25OHD concentrations, policy advice and guidelines should specify the form and/or the form specific dose of vitamin D. At present, individuals taking vitamin D₂ would be expecting the same benefits as if they were taking vitamin D₃, which is clearly not the case.

8.2.3.1 Fortification
Food fortification represents an opportunity to increase the vitamin D supply on a population-wide basis. This study showed that both orange juice and biscuits are safe and feasible vehicles for fortification with vitamin D, neither food limiting the availability of vitamin D over the other. However, in this study the focus was on fortification of food items that would not be considered staple foods for everyone; thus posing the problem that it would not increase the vitamin D supply in non-consumers.

Further research is urgently needed to develop safe, effective and sustainable solutions to prevent vitamin D deficiency and improve vitamin D related health outcomes using a food approach with particular focus on staple foods, particularly staple foods within ethnic minority groups. It has been suggested that several fortified carriers with low concentrations are a better option for fortification as a policy, because if only a few foods were fortified with high vitamin D doses, the risk of vitamin D toxicity may be higher for those who consumer larger quantities of these foods (Brown et al. 2013). This is also a reason why vitamin D₂ may actually be warranted as a suitable form of vitamin D for fortification, at present, and so fortification studies should certainly still include both vitamin D₂ and vitamin D₃.
8.2.4 Industry
There is no mandatory vitamin D fortification policy in place for any foods in the UK, following the cessation of the fortification of margarine in 2014. Nonetheless, several manufacturers have begun to voluntarily fortify their foods with vitamin D as a result of consumer demand, and the number of products on supermarket shelves is a growing market. The results of this study are of importance for food manufacturers as they show that the nutritional composition of the fortified food did not affect the availability of either vitamin D$_2$ or vitamin D$_3$, in the case of an orange juice compared with a biscuit. Manufacturers also need to be aware of the difference responses seen to vitamin D$_2$ and vitamin D$_3$, as it may determine which form they choose to fortify with. At present, there is no legal requirement for manufacturers to display the form of vitamin D used to fortify their product with on the labeling. These results suggest that it would be appropriate if the form were displayed so that the consumer could be aware.

8.2.5 Individuals
The findings from the D2-D3 Study have important implications for individuals, who should be made aware of the differences in effectiveness of the two forms at raising 25OHD concentrations, as both forms of vitamin D are available as supplements that can be bought over the counter and are currently considered equally effective. For the sake of vegans who may not want to use vitamin D$_3$ (as it is sourced from sheep’s wool – namely Lanolin), it would be important to ensure it was made clear to the general public that although vitamin D$_3$ was more effective, both forms did raise 25OHD concentrations. Interestingly, there is now a source of vitamin D$_3$ that would be suitable for vegans called Vitashine (Vegetology), obtained from a plant source called Lichen, although it is very important to note that clinical trial data using this as a source of vitamin D$_3$ are not available to assess the safety and efficacy of this source.

One problem with encouraging or supporting sourcing vitamin D from supplements or fortified foods is that, unlike sunlight, it will not promote outdoor exposure and therefore physical activity, which is likely to have additional benefits for individuals such as improved fitness and psychological wellbeing.
8.3 Critical Evaluation of the Study and Areas for Future Work

8.3.1 Study Design

Originally, the D2-D3 Study was designed to provide a daily dose of 10 μd/d (400 IU/d), but was increased to 15 μd/d (600 IU) based on the IOM (US) recommendations that were released shortly before the study trial started. Having two doses of vitamin D was also explored as an option within the study design, however, time and cost limitations meant that was not possible.

Another amendment that was made to the original study design was to have all participants consuming both an orange juice and a biscuit, as opposed to just having the product that was fortified. This meant that both participants and researchers remained blind to the intervention vehicle of fortification. This also meant that only one placebo cohort was needed. However, in hindsight, maintaining two placebo cohorts would have been beneficial for matching study numbers when the juice and biscuit cohorts were combined within the vitamin D$_2$ and vitamin D$_3$ interventions.

8.3.2 Study Recruitment

As an approach to recruitment for the study, contacting previous participants from the D-FINES study was less successful than hoped. This was partly due to several of the participants, particularly the South Asian participants, being on vitamin D supplements prescribed by their GP. The most successful method of recruitment of the Caucasian women was via the Primary Care Research Network (PCRN) mail-outs through GP surgeries, whereas for the South Asian women frequent liaison with Asian women’s centers in the local area was the most successful way of recruitment.

The study was split over two winter periods; Oct 2011-Mar 2012 and Oct 2012-Mar 2013 allowing for evaluation of recruitment techniques after the first year to assess where efforts should be placed in the second year. In the first year, 26% of the required participants had been recruited, largely due to limited time before the start of the trial to focus on recruitment as changes to the study design meant having to go back to the Ethics Committee for approval (hence recruitment was on hold at this time). It was between the first and second year that the help of the PCRN was enlisted, and more recruitment measures targeted at South Asian women were invested in, including attending mosques events, summer fetes, contacting more women’s centers and using media targeted at South Asians.
It must be kept in mind that participants volunteering to take part in the clinical trial may differ from those who do not take part. It is likely that those who volunteered are more concerned about their overall health, and therefore are likely to represent healthier cohorts than those who do not take part in trials. This study also had several exclusion criteria, including a BMI >30kg/m² and several medications including high cholesterol and high blood pressure medication. This meant that the study populations were of relatively good health, limiting extrapolation of the findings. As the study only recruited South Asian and Caucasian women this also limits the ability to extrapolate any findings to other ethnic groups, males or different age groups.

8.3.3 Study Protocol

The study protocol, outlined in Chapter 2, was adhered to throughout the study. However, there was a drop-out rate of 5% in the Caucasian and 30% in the South Asian cohorts. This was lower than the 10% expected in the Caucasian, but higher than the 20% expected in the South Asian. Seventeen women were lost to follow up, and the majority of these were South Asian. It could be that a lack of rapport was built with these women who were lost to follow-up, as some of the study mornings were very busy which resulted in less time for general conversion. English was the second language to most of the South Asian women lost to follow up, so perhaps having Urdu/Arabic interpreters at appointments would have been beneficial for rapport building, communication of information, and thus improving retention rates.

Compliance was assessed by asking participants to bring in their remaining juice tins and biscuits that had not been consumed, as opposed to asked participants to keep hold of their empty juice tins and biscuit packets. Participants were also encouraged to be honest about their compliance, for the integrity of the science, and were reassured if they had missed products. This approach appeared to be effective and 94% compliance was achieved, although understandably researchers cannot always have control over participants’ actions on an intervention such as this.

Dietary intakes of vitamin D and UVB exposure were both assessed in this study, both with limitations in their methods. Dietary intakes of vitamin D were assessed by use of a four-day food diary. Although these have been validated for use, they do have many limitations in assessing vitamin D intake. Firstly, under-reporting is common, but also four days may not be representative of vitamin D intake in particular as there are limited foods with naturally occurring vitamin D. With increasing numbers of products on the supermarket shelves being fortified with vitamin D, the vitamin D intake from fortified foods may not be accurately detected from food diaries, unless the participant
specified the brand and the researcher could input the fortified vitamin D independently. For sunlight exposure, the dosimeter badges posed some issues as some badges were lost or simply not returned. To improve the return of the badges more investigator time could have been invested in contacting to participants to ensure badges were returned or replaced where lost.

The 12-week duration of this study was too short for changes in bone density to be seen in response to the vitamin D, hence a pQCT scan was only completed at the beginning of the study. However, if further funding becomes available then bone turnover markers (CTX) will be measured in this cohort to see whether they change in response to supplementation, as it would be expected that they would, in line with the differences seen in the parathyroid hormone concentrations.

8.3.4 Laboratory Assessments of Metabolites and VDBP

The measurement of 25OHD, 25OHD$_2$ and 25OHD$_3$ was completed using LC-MS/MS at a DEQAS approved laboratory (Dr Jacqueline Berry, University of Manchester), which is the gold-standard for this measurement. However, 1,25(OH)$_2$D was measured using a chemiluminescent immunoassay (DiaSorin LIAISON) which does not allow for the differentiation between 1,25(OH)$_2$D$_2$ and 1,25(OH)$_2$D$_3$. This method was selected due to cost and time limitations; however only a sub-set of participants were included, and sufficient samples have been stored for measurement using LC-MS/MS should the funding become available at a later data.

An immunoassay kit (R&D Systems) was used to measure vitamin D binding protein in a sub-set of participants. However, following completion of this lab work, concerns about the accuracy of this kit, particularly within and between ethnic minorities, have arisen (Bouillon et al. 2014; personal communication with Dr Kerry Jones, MRC-HNR Cambridge).

8.3.5 Study Power

The D2-D3 Study was designed and powered to assess the effects of vitamin D$_2$ and vitamin D$_3$ on 25OHD concentrations. The gene expression and genetic variants analyses were secondary aims to this and therefore study numbers were powered for genetic analysis. As discussed within each genetics Chapter, study numbers are a limitation in the interpretation of results. The genetic variant/SNP data would be useful in a consortium of data as far larger samples sizes are required, however the gene expression data would benefit from higher numbers by extracting RNA for a larger proportion of the D2-D3 Study participants and funding for this is currently being sought.
8.4 Overall Conclusions

This programme of research was designed to take a holistic approach, by examining not only how vitamin D$_2$ and vitamin D$_3$, delivered via fortified foods, affect 25OHD concentrations, but also looked at how this may impact on bone health, CVD risk, as well as gene expression and therefore the metabolic and cellular pathways affected by vitamin D. In addition to investigating how the vitamin D affects individuals, the effect of individuals genetic variants on response to the vitamin D was also investigated.

In conclusion, the D2-D3 study showed that although both vitamin D$_2$ and vitamin D$_3$ delivered via fortified foods (in a dose of 15 ug/d (600 IU/d)) raise total 25OHD concentration over 12-weeks, vitamin D$_3$ leads to a greater increase in total 25OHD. Concomitantly, it has a much greater capacity to achieve ‘sufficient’ (>50 nmol/l) 25OHD concentrations across both UK dwelling South Asian and Caucasian female populations. Vitamin D$_3$ is also more beneficial to PTH concentrations (and therefore potentially to bone health), than vitamin D$_2$, but there were no differences between the two forms of vitamin D on risk of CVD. Early analysis of gene expression data has shown that the two forms of vitamin D lead to different changes in gene expression, which requires further interpretation before it can be clear whether there are differences that can be deemed as more or less beneficial to health. And finally, the genetic variant analyses showed inconsistent results and it became clear that study numbers are a considerable limitation to this data set.

Despite any limitations, this PhD work has made a novel contribution in terms of examining the comparative efficacy of vitamin D$_2$ vs. vitamin D$_3$ on 25OHD concentrations in more detail and in a larger sample size than has been undertaken before, and in an ethnic group that this has never been investigated with respect to vitamin D$_2$ vs. D$_3$. It has also, for the first time, examined changes in gene expression in response to both vitamin D$_2$ and vitamin D$_3$. This Thesis has added valuable data to the field of vitamin D food fortification, which may one day become a public health policy to help the UK population achieve the newly proposed 10 μg/d dietary vitamin D.
References


References


References


References


References


NHMRC (National Health and Medical Research Council) (2006) Nutrient reference values for Australia and New Zealand. Available at:


References


Appendices

Appendix 1 – List of all abstracts and conference proceedings

Appendix 2 – Abstract: 8th International Symposium on Nutritional Aspects of Osteoporosis 2012. Influence of Habitual Dietary Intake and Age on Risk of Poor Bone Health in Pre-Menopausal Women

Appendix 3 - Abstract: 15th Vitamin D Workshop 2012. Vitamin D2 vs Vitamin D3 Food Fortification: Preliminary Baseline Results From A Randomised Controlled Trial in Caucasian/South Asian Women - The D2-D3 Study


Appendix 7 - Abstract: BORS/BCOS Conference, 2013 Volumetric Bone Mineral Density and Dietary Patterns Across Three Age Groups of Caucasian and South Asian Women

Appendix 8 - Abstract: Vitamin D and Human Health: from the Gamete to the Grave, 2014. The Effect of Vitamin D3 Supplementation on 25OHD Status, Blood Pressure and Blood Lipid Concentrations: A Pre-Menopausal vs. Post-Menopausal Comparison

Appendix 9 - Abstract: Vitamin D Workshop, 2014. Does the presence of the metabolic syndrome impair the response to vitamin D fortification? A sub-analysis of the D2-D3 Study


Appendix 12 - Abstract: Vitamin D Workshop, 2015. Ethnic variation in the associations between genetic variants and 25-hydroxyvitamin D: The D2-D3 Study


Appendix 15 – GP letter for participant recruitment

Appendix 16 – Summary participant information sheet sent with GP letter for recruitment

Appendix 17 – Health and lifestyle questionnaire (screening questionnaire)

Appendix 18 – NHS National Research Ethics Service (NRES) Approval letter – July 2011

Appendix 19 – NHS NRES Approval letter – August 2011

Appendix 20 – NHS NRES Approval letter – September 2011

Appendix 21 – NHS NRES Approval letter – November 2011


Appendix 23 – University of Surrey Ethics Committee (EC) – August 2011

Appendix 24 – University of Surrey Ethics Committee (EC) – September 2011

Appendix 25 – University of Surrey Ethics Committee (EC) – November 2011

Appendix 26 – University of Surrey Ethics Committee (EC) – June 2012

Appendix 27 – Study Protocol: D2-D3 Study

Appendix 28 – D2-D3 Main Study Consent Form

Appendix 29 – Diet Diary Instructions

Appendix 30 – Diet Diary Sample Sheet

Appendix 31 – Adverse Events and Compliance Interview – Week 6 and 12

Appendix 32 – Diet diary analysis standard operating procedure (SOP)

Appendix 33 – Sub Study Participant Information Sheet

Appendix 34 – Sub Study Participant Consent Form
Appendix 35 - MetaGore Pathway: 'cell adhesion integrin inside-out signaling in neutrophils'

Appendix 36 - MetaCore Quick Reference Guide

Appendix 37 - MetaCore Pathway: 'Development Slit-Robo signaling'

Appendix 38 - MetaCore Pathway: 'cell adhesion - role of CDK5 in cell adhesion

Appendix 39 - MetaCore Pathway: 'immune response IL-5 signaling'

Appendix 40 - MetaCore Pathway: 'transcription assembly of RNA polymersae II pre-initiation complex on TATA-less promoters

Appendix 41 - MetaCore Pathway: 'immune response PIP3 signaling in B lymphocytes'

Appendix 42 - Metacore Pathway: 'transcription Sin3 and NuRD in transcription regulation

Appendix 43 - D-FINES: Sub-Study Participant Consent Form

Appendix 44 - LGC Genomics (UK) DNA Extraction Protocol (included with permission)

Appendix 45 – LGC Genomics (UK) SNP Genotyping Protocol (included with permission)
List of Abstracts and Conference Proceedings from the D2-D3 Study
in date order: April 2011 - December 2015

1. Tripkovic et al. (2012) 8th International Symposium on Nutritional Aspects of Osteoporosis, 17-19 May '12 (Lausanne, Switzerland) Comparison of Vitamin D2 versus Vitamin D3 supplementation in raising serum 25(OH)D status: a systematic review and meta-analysis *Oral Presentation

2. Wilson et al. (2012) 8th International Symposium on Nutritional Aspects of Osteoporosis, 17-19 May '12 (Lausanne, Switzerland): Influence of Habitual Dietary Intake and Age on Risk of Poor Bone Health in Pre-Menopausal Women *Poster Presentation

3. Tripkovic et al. (2012) 15th Vitamin D Workshop 19-22 June '12 (Texas, USA) A systematic review & meta-analysis of the comparison between vitamin D2 vs. vitamin D3 supplementation in raising serum 25(OH)D status *Poster Presentation

4. Wilson et al. (2012) 15th Vitamin D Workshop 19-22 June '12 (Texas, USA): Vitamin D2 vs Vitamin D3 Food Fortification: Preliminary Baseline Results From A Randomised Controlled Trial in Caucasian/South Asian Women - The D2-D3 Study *Poster Presentation

5. Wilson et al. (2012) FHMS Festival of Science 3rd July '12 (University of Surrey, UK): Vitamin D2 vs Vitamin D3 Food Fortification: Preliminary Baseline Results From A Randomised Controlled Trial In Caucasian/ South Asian Women - The D2-D3 Study *Poster Presentation


10. Tripkovic et al. (2013) Biosciences KTN ECR Food Sector, 23rd May '13 (London, UK) Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in Raising 25OHD Status in Caucasian & South Asian women and Mechanisms of Action (The D2-D3 Study) *Poster Presentation - Prize Winner
11. Wilson et al. (2013) *Biosciences KTN ECR Food Sector, 23rd May ’13 (London, UK)* Vitamin D Intakes and Blood Pressure in Caucasian and South Asian women *Oral Presentation - Prize Winner*

12. Wilson et al. (2013) *FHMS Festival of Science, July ’13 (Surrey, UK)* Investigating The Relationship Between Blood Pressure & Dietary Intakes Of Vitamin D In Healthy Caucasian & South Asian Women In The UK *Poster Presentation*


18. Wilson et al. (2014) *4th Postgraduate Research Conference, Feb ’14 (University of Surrey, UK)* Investigating associations between vitamin D intakes and blood pressure in Caucasian and South Asian women: baseline analysis of the D2-D3 Study *Oral Presentation*

19. Tripkovic et al. (2014) *Vitamin D and Human Health: from the Gamete to the Grave, April ’14 (Queen Marys University London, UK)* Use of diverse food matrices do not affect the bioavailability of vitamin D3 and subsequent 25OHD status in women *Poster Presentation*

20. Wilson et al. (2014) *Vitamin D and Human Health: from the Gamete to the Grave, April ’14 (Queen Marys University London, UK)* The Effect of Vitamin D3 Supplementation on 25OHD Status, Blood Pressure and Blood Lipid Concentrations: A Pre-Menopausal vs. Post-Menopausal Comparison *Poster Presentation*

21. Tripkovic et al. (2014) *Vitamin D Workshop, June ’14 (Chicago, USA)* The D2-D3 Study: Comparing the efficacy of 600IU/d vitamin D2 vs vitamin D3 in raising serum 25OHD levels *Oral Presentation*

22. Wilson et al. (2014) *Vitamin D Workshop, June ’14 (Chicago, USA)* Does the presence of the metabolic syndrome impair the response to vitamin D fortification? A sub-analysis of the D2-D3 Study *Poster Presentation*
23. Wilson et al. (2014) FHMS Festival of Research, 10th Jul ’14 (University of Surrey) Does the presence of the metabolic syndrome impair the response to vitamin D fortification? A sub-analysis of the D2-D3 Study *Oral Presentation 2 minute data blitz and Poster Presentation

24. Wilson et al. (2014) FHMS Festival of Research, 10th Jul ’14 (University of Surrey) The Effect of Vitamin D3 Supplementation on 25OHD Status, Blood Pressure and Blood Lipid Concentrations: A Pre-Menopausal vs. Post-Menopausal Comparison *Poster Presentation


28. Wilson et al. (2014) BBSRC DRINC 11th Dissemination Meeting 8-9 Oct ’14 (Chester, UK) A systems biology approach to studying the effect of increasing vitamin D intake through food fortification on 25(OH)D status in different population groups: The D2-D3 Study *Oral Presentation

29. Tripkovic et al. (2014) National Osteoporosis Society Conference, Nov ’14 (Birmingham, UK) Daily supplementation with vitamin D3 is comprehensively more effective than vitamin D2 in raising 25OHD status and concomitantly reducing parathyroid hormone levels: Implications for bone health *Oral Presentation *Young Investigator Award


32. Wilson et al. (2015) University of Surrey Postgraduate Research Conference, April ’15 (Surrey, UK) Is vitamin D3 more effective than vitamin D2 in raising 25OHD status in women with osteoporosis and osteopenia? *Poster Presentation

33. Tripkovic et al. (2015) International Symposium on the Nutritional Aspects of Osteoporosis, June ’15 (Montreal, Canada) Baseline 25-hydroxyvitamin D influences the total change in 25-hydroxyvitamin D in response to 15µg/600IU daily vitamin D2 or D3 *Poster Presentation

35. Tripkovic et al. (2015) *Musculoskeletal Health in the 21st Century, June ’15 (University of Surrey, UK)* Baseline 25-hydroxyvitamin D influences the total change in 25-hydroxyvitamin D in response to 15μg/600IU daily vitamin D2 or D3 *Poster Presentation


38. Tripkovic et al. (2015) *FHMS Festival of Research, Jul ’15 (University of Surrey)* Baseline 25-hydroxyvitamin D influences the total change in 25-hydroxyvitamin D in response to 15μg/600IU daily vitamin D2 or D3 *Poster Presentation

39. Wilson et al. (2015) *FHMS Festival of Research, Jul ’15 (University of Surrey)* Associations between genetic variants and vitamin D status in Caucasian and South Asian women: The d2-D3 Study *Poster Presentation

Influence of habitual dietary intake and age on risk of poor bone health in pre-menopausal women

The effect of habitual diet and age on bone health has been extensively researched in post-menopausal women, but little data is available for pre-menopausal women. At present it is unclear as to whether young and middle-aged women are at a tangible risk of poor bone health and if so, what factors may be influencing this perceived risk prior to the menopause. The current study aimed to assess habitual dietary intake and volumetric bone mineral density (vBMD) in healthy weight, pre-menopausal women.

In total 41 healthy, pre-menopausal women of normal weight (BMI 20-25kg/m²), aged 20-49 years were recruited as part of an RCT trial, with cross sectional analysis performed for this specific sub-study. The women were divided into two groups - aged 20-34 years (n=20) and 35-49 years (n=21). Nutrient intake was assessed with a 4 day estimated food diary and vBMD was measured via peripheral Quantitative Computed Tomography (pQCT). Anthropometrics, including body mass index (BMI) and blood pressure were also recorded.

For the cohort analysed as a whole (n 41, aged 33.7±10.7yrs, BMI 21.5±2.2 kg/m²) no significant correlations were found between habitual dietary intake and vBMD, nor was there a significant relationship between age and vBMD.

The younger age category (n 20, 24.0±3.50yrs, BMI 20.7±1.90 kg/m²) showed few associations between diet and bone health, except for a significant positive association between Stress Strain Index (SSI) and vitamin D intake (P<0.009); this is despite relatively low mean intake values for vitamin D within the group (2.49±2.46µg/day).

The older age group (43.8±4.03yrs BMI 22.3±2.24 kg/m²) showed a strong significant association between age and both total density (P<0.024), and the T Score for total density (P<0.023). Vitamin D intake was not associated with any vBMD parameters, however significant associations were found between SSI and absolute daily intakes for energy (P<0.009), fat (P<0.03), carbohydrate (P<0.004), dietary fibre (P<0.01), potassium (P<0.009), calcium (P<0.04) and magnesium (P<0.02).

When comparing between the age groups, a significant difference was found for both total density (P<0.05) and the T Score for total density (P<0.05).

This study demonstrates that despite the relatively young age of the cohort, risk to poor bone health increases steadily with age. The influence of dietary intake on bone density in the older premenopausal women showed some interesting associations and further research is warranted.
Evidence suggests that in the UK, South Asian women are vitamin D-deficient throughout the year and Caucasian women are at risk of vitamin D insufficiency and deficiency during the winter months. The D2-D3 Study (a food fortification intervention trial, funded by BBSRC DRINC Programme [No. BB/I006192/1]) has primarily been set-up to compare the efficacy of 15µg/d [600IU/d] Vitamin D2 vs. 15µg/d Vitamin D3 in raising serum 25(OH)D levels in both Caucasian and South Asian women above the ‘deficiency/insufficiency’ thresholds (25nmol/l and 40nmol/l respectively) during the winter months (Tripkovic et al 2012). The aim of this preliminary study was to examine baseline characteristics for diet/bone associations. For the completed first cohort, participant characteristics are as follows: n88 women recruited (n71 Caucasian, n17 South Asian), age 40.3±13.2years, BMI 23.8±3.5kg/m², Body Fat 28.8±7.8%, waist 78.5±11.5cm. When comparing between ethnicities, Caucasian women were significantly taller (p<0.001) and yet had a lower body fat % (p=0.04) than the South Asian women. Volumetric bone mineral density (vBMD) was measured via peripheral quantitative computed tomography (pQCT), with no significant differences found between the ethnic groups. Dietary intakes were markedly different, with Caucasian women consuming significantly greater amounts of energy, saturated fat, polyunsaturated fat, trans fats, fiber, alcohol, calcium, magnesium and sodium than the South Asian women (p<0.04). Vitamin D intake was low for all participants, with an average intake of 2.6±2.0µg per day. To date, this analysis of cross-sectional data appears to show little evidence linking body composition and dietary intake to bone density taking into account ethnicity. Our on-going longitudinal data will quantify the relationship between these factors further. Tripkovic et al AJCN 2012 (in press).
The influence of habitual dietary intake on bone density in pre-menopausal women

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To date, extensive research has been conducted examining the effect that ageing and the habitual diet may have on the bone health of post-menopausal women\(^1,2\) however, little data is available for pre-menopausal women\(^3\). At present it is unclear as to whether young and middle-aged women are at a tangible risk of poor bone health and if so, what factors may be influencing this perceived risk prior to the menopause. The current study aimed to assess habitual dietary intake and volumetric bone mineral density (vBMD) in pre-menopausal women.

In total 41 healthy, pre-menopausal women of normal weight (BMI 21.5±2.2 kg/m\(^2\)), aged 34.1±10.7 years were recruited as part of an RCT trial, with cross sectional analysis performed for this specific sub-study. The women were divided into two groups – aged 20–34 years (n = 20) and 35–49 years (n = 21). Nutrient intake was assessed with a 4-day estimated food diary and vBMD was measured via peripheral Quantitative Computed Tomography (pQCT).

For the cohort analysed as a whole (n = 41), no significant correlations were found between habitual dietary intake and vBMD, nor was there a significant relationship between age and vBMD. The younger age category (n = 20, 24.0±3.5 years, BMI 20.7±1.90 kg/m\(^2\)) showed few associations between diet and bone health, except for a significant positive association between Stress Strain Index (SSI) and vitamin D intake (P < 0.009) Fig. 1; this is despite relatively low mean intake values for vitamin D within the group (2.49±2.46 μg/day).

The older age group (43.8±4.03 yrs BMI 22.3±2.24 kg/m\(^2\)) showed a strong significant association between age and both total density (P < 0.024), and the T Score for total density (P < 0.023). Vitamin D intake was not associated with vBMD, however significant associations were found between SSI and absolute daily intakes for energy (P < 0.009), fat (P < 0.03), carbohydrate (P < 0.004), dietary fibre (P < 0.01), potassium (P < 0.009), calcium (P < 0.04) and magnesium (P < 0.02). When comparing between the age groups (Fig. 2), a significant difference was found for both total density (P < 0.05) and the T Score for total density (P < 0.05).

This study demonstrates that the influences on bone health are difficult to discern when focussing solely on dietary intake, especially for the younger premenopausal women. However, the influence of dietary intake on bone density in the older premenopausal women showed some interesting associations and further research is warranted.

This work was funded by the BBSRC DRINC Programme (Grant No.BB/I006192/1). The views expressed are those of the authors alone.

Association between dietary vitamin D intakes and blood pressure in Caucasian and South Asian females: preliminary analysis of the D2–D3 Study

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There is evidence of an inverse association between vitamin D status and blood pressure(1), which indicates a potential role for vitamin D in cardiovascular disease prevention. The work to date has been particularly focused on 25(OH)D levels(2), but little research has focused on the influence of dietary intake of vitamin D on blood pressure measurements, especially in population groups at risk of vitamin D deficiency. Therefore, the aim of this study was to investigate and compare the association between dietary vitamin D intake and blood pressure in women of both Caucasian (Cn) and South Asian (SA) origin, taking into consideration other dietary influences and anthropometrics.

A total of 88 women (n71 Cn, n17 SA) aged 20–64 years were recruited from the Surrey area as part of the D2-D3 study (a vitamin D food fortification trial). As part of their baseline visit for the D2-D3 study, participants completed a four-day food diary and had anthropometric and blood pressure measurements taken. The four-day food diaries were analysed using DietPlan6 to determine nutrient intakes. Blood pressure was measured in rested participants and the mean of three measurements was taken for systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and heart rate (HR). Analysis was carried out as whole group, between ethnic groups and also between age groups (20–34, 35–49 and 50–64 years) and body mass index (BMI) categories (18–24.9 kg/m² and 25–30 kg/m²).

The overall mean age and BMI (n88) were 40.3±13.2years and 23.8±3.5 kg/m² respectively. Compared to SA women, the Cn women had a significantly lower body fat percentage (SA: 31.8±6.6 vs. C: 28.1±6.6, p = 0.046) and waist-hip ratio (SA: 0.80±0.07 vs. C: 0.76±0.07, p = 0.02) but there were no differences in age or BMI.

Although dietary intakes were markedly different between ethnic groups (data not presented), vitamin D intakes were not significantly different (Cn: 2.7±1.9 µg/d vs. SA: 2.2±2.7 µg/d). The average intake of vitamin D was 2.6±2.0 µg/d (105.2±81.4 IU/d). The results show that dietary vitamin D intake did not correlate with blood pressure measurements in the group as a whole nor in any sub-group analysis taking into account BMI and age.

Further analysis into the blood pressure measurements showed that Caucasian women had a significantly higher PP (Cn:39.1±8.4 vs. SA:33.8±8.8, p = 0.024); but there were no significant differences in SBP, DBP, MAP or HR. SBP consistently increased with age, body weight, BMI and waist circumference (p<0.001); as did DBP (p<0.001); and MAP (p<0.001) which was consistent in sub-group age analysis, except in women aged 50–64 years. PP increased only with age (p<0.001) and HR increased only with weight (p = 0.043).

This works confirms the association between age and BMI and blood pressure(3)(4), but did not find any correlation between dietary vitamin D intakes and blood pressure at the levels seen in this sample group. Although the UK does not have a recommended dietary intake (RDI) for men and women aged 5–64 years, there is an RDI of 10 µg/d (400 IU/d) set for those who are at risk of deficiency. As the average intake of vitamin D in this study was far below what is considered adequate to support essential metabolic pathways, it would be valid and interesting to investigate whether a higher and sustained level of vitamin D intake can positively influence blood pressure with the potential consequence of ameliorating CVD risk.

Vitamin D intakes and blood pressure in Caucasian and South Asian women aged 20–64 years – baseline analysis of the D2–D3 study

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High blood pressure affects 40% of adults worldwide and is one (preventable) contributing factor in the complex aetiology of cardiovascular disease. In cross-sectional studies, an inverse association between vitamin D status [25(OH)D levels] and blood pressure has been shown\(^1,2\), and recent research has indicated that supplementation with vitamin D can lead to a significant reduction in systolic blood pressure, in a dose dependant manner\(^3\). Our recent findings\(^4\) investigating associations between dietary intakes of vitamin D and blood pressure measures showed no associations, but were based on a small population number (n 88). Therefore, the aim of this study was to investigate and compare the association between dietary vitamin D intake and blood pressure in a larger population of women from both Caucasian (Cn) and South Asian (SA) communities, taking into consideration anthropometrics and other dietary influences.

A total of 335 women (n 245 Cn, n 90 SA) aged 20–64 years were recruited from the Surrey area as part of the D2–D3 study (Vitamin D Food Fortification Trial comparing Vitamin D2 vs. Vitamin D3). Participants had anthropometric and blood pressure measurements conducted, and also completed a four-day food diary, which was analysed using DietPlan6. Blood pressure measurements were taken in rested participants and the mean of three measurements were taken to determine systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), pulse pressure (PP) and heart rate (HR). Analyses were carried out as whole group and between ethnic/age groups (20–34, 35–49 and 50–64 yrs) and body mass index (BMI) categories (18–24.9 kg/m\(^2\) and 25–30 kg/m\(^2\)).

The mean age and BMI of the study population (n335) were 43.6 ± 12.3 years and 24.1 ± 3.8 kg/m\(^2\) respectively, and the mean SBP, DBP, MAP, PP and HR were 118 ± 15 mmHg, 78 ± 10 mmHg, 92 ± 11 mmHg, 40 ± 9 mmHg and 68 ± 10 bpm respectively.

When comparing anthropometrics and blood pressure measurements between ethnic groups, the SA women had a significantly lower age (SA:37.3 ± 10.4 yrs vs. Cn:46.0 ± 12.1 yrs, P<0.001), but higher BMI (SA:25.3 ± 4.4 vs. Cn:23.6 ± 3.5, P<0.001), body fat percentage (SA: 32.6 ± 7.0 vs. Cn:29.0 ± 6.3, p = 0.000) and waist-hip ratio (SA:0.84 ± 0.08 vs. Cn:0.79 ± 0.07, P<0.001) than the Cn women. Despite these differences in anthropometrics, the SA women had lower SBP (SA:113 ± 13 vs. Cn:120 ± 15 P<0.001), PP (SA:36.5 ± 7.4 vs. Cn:41.4 ± 8.8 P = 0.000) and MAP (SA:88.6 ± 10.5 vs. Cn:92.5 ± 10.8 P<0.003) than Cn women, but higher HR (SA: 70.7 ± 9.0 vs. Cn:67.6 ± 9.6 P<0.008).

Dietary intakes did vary between the ethnic groups, although macro-nutrient intakes were not significantly different (data not presented). Vitamin D intakes were also not significantly different between the groups (SA:2.2 ± 2.1 μg/d vs. Cn:2.7 ± 2.3 μg/d). These dietary vitamin D intakes showed no correlation to any measures of blood pressure when analysing the different groups or sub-group analysis taking into account ethnicity/BMI/age.

This work highlights the low dietary vitamin D intakes in both Cn and SA women in the UK. At these low levels, dietary vitamin D intakes show no correlation to blood pressure measures. Our results, alongside RCTs showing an effect of supplementary vitamin D on blood pressure, suggest that intakes of vitamin D at levels achievable without the use of supplementation, are not sufficient to influence blood pressure. Further analysis is underway in the D2–D3 study cohort examining vitamin D status and blood pressure (baseline/change).

The D2–D3 Study is funded by the BBSRC DRINC Programme; LW is recipient of a BBSRC PhD Scholarship.

VOLUMETRIC BONE MINERAL DENSITY AND DIETARY PATTERNS ACROSS THREE AGE GROUPS OF CAUCASIAN AND SOUTH ASIAN WOMEN

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It is well established that low bone mass is the most important measurable determinant of osteoporotic fractures. Studies have shown an age-related decrease in bone mineral density (BMD) in both men and women[1-2]. However in women rapid bone loss occurs predominantly during the menopausal transition. The objective of the study was to assess volumetric BMD (vBMD) and dietary intakes across three age groups (A:20-34yrs, B:35-49yrs, C:50-65yrs) of healthy women.

For this cross-sectional analysis, 335 women (n245 Caucasian, n90 South Asian) completed a baseline visit as part of The D2-D3 Study (a BBSRC DRINC funded vitamin D food fortification trial). Data collected included anthropometrics, a four-day food diary and a peripheral quantitative computed tomography (pQCT) scan at the distal and mid-shaft sites of the radius.

Group A (n86), B (n134) and C (n115) had a mean age of 27.1±4.5yrs, 42.9±4.3yrs and 56.9±4.4yrs, and mean BMI of 23.1±4.3kg/m^2, 24.0±3.4kg/m^2 and 24.8±3.7kg/m^2 respectively. BMI was significantly lower in group A than both B and C (p<0.001).

At the distal site, group C had significantly lower total vBMD than both A and B (C:299±50mg/cm^3, A:321±48mg/cm^3, B:323±46mg/cm^3, p<0.001), and a lower trabecular vBMD than group A (C:176±34mg/cm^3, A:188±31mg/cm^3, p<0.008). At the mid-shaft site, cortical vBMD was significantly different between all three groups, with density decreasing as age increased (A: 1131±55 mg/cm^3, B:1130±147 mg/cm^3, C:1107±107mg/cm^3,p<0.016). Groups A and B also had significantly higher T-Scores than group C (A:-1.08±0.80, B:-1.03±0.77, C:-1.45±0.84, p<0.006).

In group A, compared to both B and C, there was a significantly lower daily intake of protein (A:66±16g, B:74±18g, C:74±17g, p<0.007), potassium (A:2580±718mg, B:3039±929mg, C:3316±1106mg, p=0.001) and vitamin K(A35.8±57.2µg, B:50.9±70.4µg, C:50.8±47µg, p<0.01). Group A, compared to group C only, also had a lower daily intake of magnesium (A:258±75mg, C:309±101mg, p=0.01) and phosphorus (A:1156±308mg, C:1293±263mg, p=0.004). Alcohol intake was significantly higher in group C, than both A and B (C:13.2±12.0, A:5.6±10.1g, B:9.1±12.2g, p<0.003).

Despite higher intakes of nutrients known to have a positive role in skeletal health (protein, magnesium, phosphorus), the eldest age group had lower vBMD supporting the impact of age-related bone loss over the influence of dietary intake, on vBMD. Further research is underway examining the relationship between energy-adjusted nutrient intakes and bone health.

The D2-D3 Study is funded by the BBSRC DRINC Programme; LW is recipient of a BBSRC PhD Scholarship.

The Effect of Vitamin D3 Supplementation on 25OHD Status, Blood Pressure and Blood Lipid Concentrations: A Pre-Menopausal vs. Post-Menopausal Comparison

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BACKGROUND Epidemiological research has shown a close association between vitamin D deficiency (25OHD <25nmol/l) and cardiovascular mortality and morbidity. However, a causal relationship between vitamin D status and cardiovascular disease (CVD) risk factors, such as hypertension or an atherogenic lipid profile, has not been established. Post-menopausal (PoM) women are at an increased risk of CVD compared to pre-menopausal (PrM) women. Therefore this study aimed to compare the response to vitamin D3 supplementation between PrM and PoM women, with respect to changes in 25OHD status and key CVD risk factors.

METHODS A total of 146 Caucasian women (n90 PrM, n56 PoM) were recruited onto the D2-D3 Study and randomised to receive either placebo or vitamin D3 (15μg/600IU) daily for 12-weeks. Anthropometrics, blood pressure and a fasted blood samples (for 25OHD and lipid analysis) were collected from each participant at week 0 (baseline) and week 12.

RESULTS At baseline, the PrM women had a significantly lower body mass index (BMI) and blood pressure (BP) than the PoM women (22.9±3.1 vs 24.7±3.8kg/m2, 116±12/76±9mmHg vs 128±17/83±10mmHg respectively; p<0.001), and significantly lower cholesterol, high-density lipoprotein (HDL) and low density lipoprotein (LDL) levels (5.1±0.9 vs 6.0±1.0mmol/l, 1.81±0.34 vs 1.91±0.46mmol/l, 2.89±0.77 vs 3.62±0.89mmol/l respectively; p<0.001).

In the placebo group there was a significant decrease in 25OHD status between baseline and week-12 in both PrM and PoM women (PrM n32: -16.5±12.8%, PoM n16: -12.7±7.0%; p<0.001), whereas in the vitamin D3 group there was a significant increase in 25OHD status (PrM n58: 28.6±20.0%, PoM n40: 32.6±25.2%; p<0.001). The percentage change in 25OHD status within each group was not significantly different between the PrM and PoM women. The placebo group also saw a significant increase in BMI and waist circumference between baseline and week 12 in PrM women only (23.7±3.4 vs 24.0±3.6kg/m2, 78.9±10.0 vs 81.0±10.2cm respectively, p<0.04), whereas in the vitamin D3 group, both PrM and PoM women had a significant increase in triglycerides (0.98±0.44 vs 1.15±0.73mmol/l, 1.11±0.53 vs 1.25±0.47mmol/l, p<0.01).

When looking at absolute change from baseline, the only correlation was a weak positive correlation between change in 25OHD status and change in BMI within the PrM women in the vitamin D3 group (28.6±20% and 0.31±2.4% respectively, r=-0.265, p<0.050)

CONCLUSION These results suggest that changes in 25OHD status, BP and blood lipids in response to vitamin D3 supplementation are not affected by menopausal status, as any significant changes were seen in both PrM and PoM groups. The increase in triglycerides within the vitamin D3 treatment group is of interest because, although the study did not shift participants above the clinically ‘at-risk’ cut off of 1.7mmol/l, the results contradict previous literature which, although non-significant, have shown a reduction in triglycerides. The data also contradicts previous data suggesting that BMI is negatively correlated with 25OHD status which warrants further investigation, however the mean change in BMI was minimal (0.31%) and the correlation was weak so although the finding was statistically significant, it may not be of clinical importance.
 DOES THE PRESENCE OF METABOLIC SYNDROME IMPAIR THE RESPONSE TO VITAMIN D FORTIFICATION? A SUB-ANALYSIS OF THE D2-D2 STUDY

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An inverse association between serum 25OHD (s25OHD) levels and the prevalence of Metabolic Syndrome (MetS) has been shown in cross-sectional data[1]. MetS is characterized by a clustering of cardiovascular disease risk factors such as abdominal obesity, hypertension and raised triglycerides. As a sub-analysis to the D2-D3 Study, we aimed to examine whether those identified as having MetS respond to both vitamin D2 and D3 as effectively as those characterized as not having MetS (NMS). A total of 335 women aged 20-64yrs, of which n27 were identified as having MetS, were randomized to receive either placebo (PL: MetS n7, NMS n58), 600IU vitamin D2 (D2: MetS n10, NMS n123), or 600IU vitamin D3 (D3: MetS n10, NMS n127) daily for 12-weeks during the wintertime. Irrespective of assigned intervention, there was no significant difference in s25OHD levels at baseline between MetS (n27) and NMS (n308) cohorts (18.1±10.6ng/ml vs 20.9±11.8ng/ml respectively), nor were there significant differences between MetS and NMS cohorts in the placebo and intervention groups (PL: 16.2±11.1ng/ml vs 21.2±10.8ng/ml, D2: 17.8±10.5ng/ml vs. 21.4±12.6ng/ml, D3: 19.6±11.3ng/ml vs 20.2±11.6ng/ml respectively). After 12-weeks, % change from baseline in s25OHD in the placebo and intervention groups were also not significantly different between MetS and NMS cohorts (PL: -22.2% vs -13.1%, D2: +68.7% vs +63.3%, D3: +123.8% vs +103.4% respectively). We also found no significant difference in the response to exogenous 25OHD2 and 25OHD3 between the MetS and NMS groups. Thus the major finding of the present analysis is that MetS did not affect response to 600IU/d of either vitamin D2 or D3.[1] Ford et al Diabetes Care May 2005 vol. 28 no. 5 1228-1230
The D2-D3 Study: comparing the efficacy of 15μg/d vitamin D2 vs. D3 in raising vitamin D status in both South Asian and Caucasian women, and the ethical implications of placebo treatment

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In the UK, the main source of vitamin D is sunlight as there are few dietary sources. Factors such as darker skin tone and a reduced exposure to sunlight due to clothing worn for cultural reasons, reduce the ability for individuals to make vitamin D from the action of UVB rays on skin. Therefore ethnic groups such as South Asian (SA) women are at a greater risk of deficiency year round, compared to white Caucasian (Cauc) women who may only be at risk during the winter months. Supplementation with vitamin D may therefore have a beneficial role in such ‘at-risk’ population groups.

The D2-D3 study aimed to examine and compare the efficacy of 15μg/d vitamin D2 vs. vitamin D3 in raising serum 25OHD (s25OHD) levels, and to assess response within and between SA and Cauc women. Participants were randomised to receive either placebo, vitamin D2 in orange juice (D2J), vitamin D2 in a biscuit (D2B), vitamin D3 in orange juice (D3J), or vitamin D3 in a biscuit (D3B) daily for 12-weeks during the wintertime. A total of 63 SA and 204 Cauc.

As shown in Table 1, baseline serum 25OHD (s25OHD) concentration of the Cauc groups were more than twice those of the SA groups. In response to vitamin D2 and D3 interventions, s25OHD significantly increased in both SA and Cauc groups (p < 0.001), but percentage increase was higher in SA due to their poor s25OHD status at baseline. Placebo treatment led to a decrease in s25OHD in both the Cauc (p < 0.001) and SA group (p = 0.081).

At baseline, 61.1% of the SA participants were vitamin D ‘deficient’, with s25OHD <25 nmol/l, and at the end of the 12-week intervention period 85.7% of the SA participants within the placebo group were vitamin D ‘deficient’.

This study provides evidence to show that s25OHD increases in response to either vitamin D2 or D3 in both SA and Cauc women, but that there is a greater response to vitamin D3 within both ethnic groups suggesting this may be the optimum form to use when treating deficiency. Furthermore this study data raises one key issue; whether it remains ethical to run randomised controlled vitamin D trials in SA populations, when there is a risk of receiving placebo treatment leading to greater levels of deficiency?

The D2-D3 Study is funded by the BBSRC DRINC Programme (Grant No. BB/I006192/1).


### Table 1. Baseline and week 12 s25OHD levels (nmol/l) in both SA and Cauc women

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<th>Treatment Group</th>
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<td>19.5 ± 7.7</td>
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<td>59.2 ± 23.2</td>
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<td>14</td>
<td>34.7 ± 22.7</td>
<td>52.9 ± 13.2</td>
<td>42</td>
<td>57.7 ± 26.2</td>
<td>73.9 ± 18.7</td>
<td>204</td>
<td></td>
</tr>
<tr>
<td>D2B</td>
<td>13</td>
<td>29.7 ± 23.0</td>
<td>49.4 ± 17.0</td>
<td>45</td>
<td>58.4 ± 24.7</td>
<td>73.6 ± 18.0</td>
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</table>

### Appendix 10

Summer Meeting, 14–17 July 2014, Carbohydrates in health: friends or foes
Is vitamin D3 more effective than vitamin D2 in raising 25OHD status in women with osteoporosis and osteopenia?

Wilson L (1), Tripkovic L (1), Hart K (1), Elliott R (1), Smith CP (2), Bucca G (2), Penson S (3), Chope G (3), Hyppönen E (4), Berry JL (5) and Lanham-New S (1)

Introduction: Poor vitamin D status (hypovitaminosis D) is an increasingly common problem in the UK, with prevalence highest during the winter months as a result of reduced UV exposure for dermal production of vitamin D. With the implications of a low vitamin D status known to include poor bone density, establishing strategies to improve the vitamin D status in the UK is vital. Our objective was to measure changes in serum 25OHD in response to 15µg/d vitamin D2 and D3 supplementation, in those with osteopenia and osteoporosis compared to those with normal bone density to investigate whether differing strategies may be needed.

Material and Methods: Participants were recruited as part of the D2-D3 Study, and randomised to receive either 15 µg vitamin D2 (D2) or vitamin D3 (D3) daily for 12 weeks. Vitamin D status (25OHD) was measured at baseline and week 12. Participants were characterised as having osteoporosis/osteopenia (O) or normal bone density (N) based on a T-Score from a peripheral quantitative computed tomography (pQCT) scan of the radius. These analyses are based on four treatment groups: D2N (n39), D3N (n31), D2O (n79) and D3O (n83).

Discussion: At baseline, there were no significant differences in 25OHD status between the four treatment groups (D2N: 52.21±36.0nmol/L, D3N: 46.09±26.27nmol/L, D2O: 55.67±28.56, D3O: 57.05±28.02nmol/L). In response to the 12-week vitamin D intervention, all four treatment groups had a significant increase in 25OHD status (D2N: 66.49±23.01nmol/L, D3N: 72.58±23.22nmol/L, D2O: 69.20±21.29nmol/L, D3O: 89.50±24.97nmol/L) with a higher percentage increase seen in the D3 groups (D3N: 108.86±131.41%, D3O: 103.13±139.16%) than the D2 groups (D2N: 74.09±96.84%, D2O: 46.16±63.22%). At 12-weeks the 25OHD status of the D3O group was significantly higher than all three other groups (p<0.001), despite there being no significant differences between the groups at baseline.

Conclusion: The present study provides evidence to suggest that although both vitamin D2 and D3 significantly improve 25OHD status, regardless of bone density, those with osteoporotic or osteopenic bones may respond better to supplementation with vitamin D3. These data warrant further investigation, controlling for age and baseline vitamin D status.
ETHNIC VARIATION IN THE ASSOCIATIONS BETWEEN GENETIC VARIANTS AND 25-HYDROXYVITAMIN D: THE D2-D3 STUDY.

L. Wilson, L. Tripkovic, A. Cavadino, K. Hart, CP. Smith, G. Bucca, R. Elliott, S. Penson, G. Chope, J. Berry, S. Lanham-New & E. Hyppönen Department of Nutritional Sciences, University of Surrey, Guildford, GU2 7XH, UK; University College London, London, WC1E 6BT, UK; University of South Australia, Adelaide, Australia.

Circulating levels of serum 25-hydroxyvitamin D (25OHD) are determined both by genetic and environmental factors. We investigated whether there are ethnic differences in the associations between previously reported vitamin D-related polymorphisms and serum 25OHD concentrations in healthy women recruited in Surrey (UK) as part of the D2-D3 Study. Serum 25OHD concentrations and a total of 8 single nucleotide polymorphisms (SNPs) in the GC, DHCR7, CYP2R1 and CYP24A1 genes were analyzed in 234 white Caucasian (WC) women and 81 South Asian (SA) women. Individual SNPs, GC haplotypes (determined from rs4588 and rs7041), DHCR7 risk scores (rs12785878 and rs11234027), CYP2R1 risk scores (rs10741657 and rs12794714) and CYP24A1 risk scores (rs6013897 and rs17216707) were used in the analyses. The two SNPs in the DHCR7 gene, and the related risk score were significantly negatively associated with serum 25OHD levels in the WC but not in SA women (p-interaction <0.0001). Some evidence for ethnic variation was also seen for CYP24A1 (p-interaction 0.003 for rs6013897, p-interaction 0.01 for CYP24A1 risk score) but not for GC or CYP2R1. This study highlights the potential for ethnic differences in the way genetic factors affect 25OHD concentrations, warranting further research to confirm the observed associations.
Genetic variants in the vitamin D binding protein/GC gene have been shown to strongly influence bone mineral density (BMD) and fracture risk. We examined the role of GC genotype on BMD in healthy women from two ethnic groups; white Caucasian and South Asian.

Background characteristics and a fasted blood sample were collected from 233 white Caucasian and 80 South Asian women as part of the D2-D3 Study[1] based in Surrey, UK. BMD was measured by a pQCT scan of the radius and genotyping was based on two single-nucleotide polymorphisms (rs4588 and rs7041) in the GC gene. Analyses focused on GC haplotype, as well as individual SNPs.

Genotype frequency differed between ethnic groups for rs7041 (p=0.04), but not for rs4588 or GC haplotype (p=0.84, p=0.63 respectively). In unadjusted analyses, vitamin D lowering alleles in GC haplotype and rs4588 were correlated with higher BMD (r=0.12, p=0.04 and r=0.13, p=0.02, respectively). However, when adjusted for age, weight and ethnicity in linear regression analyses this association was attenuated for both GC haplotype and rs4588 (beta=1.92, 95%CI -0.35 to 4.19, p=0.10 and beta=8.74, 95%CI -0.03 to 17.5, p=0.05, respectively). While there was no statistical evidence for ethnic differences in the association between GC and BMD (p-interaction>0.16 for all comparisons), analyses stratified by ethnic group suggested more pronounced genetic associations for Caucasian compared to South Asian women for GC haplotype (beta=2.80, 95%CI 0.37 to 5.24, p=0.03 vs. beta=-0.97, 95%CI -5.96 to 4.02, p=0.70, respectively) and rs4588 (beta=11.8, 95%CI 2.30 to 21.3, p=0.02 and beta=-0.01, 95%CI -18.9 to 18.9, p=0.999, respectively).

Although data from these analyses must be interpreted with caution due to a limited sample size, the results suggest that vitamin D status lowering alleles may be associated with a higher bone mineral density. This data could be supportive of work suggesting that despite presence of vitamin D lowering alleles, and thus lower circulating 25-hydroxyvitamin D levels, the bioavailability of vitamin D is better[2]. The data also suggest potential ethnic variation in genetic influence on bone mineral density, warranting further research in a larger sample size.

[1] Ergocalciferol vs. Cholecalciferol Food Fortification Study (ISRCTN: 23421591) funded by the BBSRC DRINC Programme (Grant No. BB/I006192/1).
Since the beginning of our new millennium, vitamin D has been the absolute focus of attention: there can be no doubt about that! Whether it be the scientific, clinical or academic communities, government/regulatory organisations, industry, media or indeed the public, everyone has ‘woken up’ to the reality that the functions of this nutrient go far wider than that of the skeletal system. Concomitant with that, there is universal acceptance that we have a high prevalence of people with vitamin D levels lower than is good for their health.

Vitamin D is a most unique nutrient – the term ‘vitamin’ is a misnomer since vitamin D is not a ‘vital-amine’ in the true sense of the word but rather it is a pro-hormone – with the main source not being diet but rather ultraviolet B-rays (UVB) from sunlight. This makes vitamin D such a challenging (but exciting!) nutrient to study as in areas of northern latitude, vitamin D can be made from UVB only during the months of April to September. Hence, randomised, controlled trials (RCTs) involving vitamin D should strictly be confined to the winter months when vitamin D is not made endogenously via the act of sunlight on skin, and all dietary vitamin D studies (cross-sectional and longitudinal) need to adjust for sunlight exposure in their analyses (Lanham-New et al. 2011).

Vitamin D₃ is formed as the direct effect of UV irradiation of the skin. The action of UVB converts 7-dehydrocholesterol to pre-vitamin D, which is then metabolised to vitamin D by a temperature-dependent isomerisation. We know that 7-dehydrocholesterol is a zoosterol, which functions in the serum as a cholesterol precursor, and is converted to vitamin D₃ in the skin, therefore operating as pro-vitamin D₃. This is particularly important since there is a growing recognition that people who take cholesterol-lowering statin drugs have a problem with vitamin D deficiency, although to date this has attracted relatively little focus. Cholesterol is required by the body to synthesise vitamin D and statin drugs are responsible for reducing cholesterol production and eliminating it, leading many to speculate that statin drug users do not have enough cholesterol to process vitamin D efficiently. Studies, albeit observational in nature, are beginning to show convincingly that statin users have a greater prevalence of vitamin D deficiency, with muscle pain being a common characteristic. This is an area that the clinical field must take forward as a genuine concern in their clinical practice and is a research area that warrants urgent attention.

Once vitamin D is metabolised from pre-vitamin D to vitamin D, it is transported via the general circulation and, following enzymatic activity in the liver (by 25-hydroxylase), it is converted to 25-hydroxy vitamin D (25OHD), which is considered to be the best clinical indicator of vitamin D status. The concentration of 25OHD in the blood reflects the vitamin D supply from both the skin and the diet, and with a decent half-life (approximately 3 weeks), it is a good integrated marker of recent vitamin D supply and can thus be used to assess vitamin D adequacy. Using the vitamin D-binding protein, 25OHD is transported to the kidney where it undergoes a final hydroxylation step via the enzyme 1-alpha-hydroxylase to become 1-alpha, 25-dihydroxyvitamin D, also known as calcitriol, which is the active form of vitamin D.

What has held the field back is the lack of standardised measurements of 25OHD status, with laboratories worldwide showing alarmingly poor consistency of measurement. Indeed, in the well-publicised paper by Binkley et al. (2004), remarkably different results were yielded from samples, which had been spiked with 20 ng/ml and processed by a number of top vitamin D measuring laboratories using their specific methodologies. This has had ramifications for the field of vitamin D research and is one clear reason why there are such inconsistencies and controversies, nationally and internationally, as to what level of 25OHD status defines...
vitamin D ‘deficiency’, ‘insufficiency’ and ‘optimum’ and which method should be used (Spiro & Buttriss 2014). However, what is very positive is that a gold standard reference vitamin D method (liquid chromatography-mass spectrometry; LC-MS) has been introduced in recent years and is now being actively used by a number of key organisations, including (but not limited to) the US Centres for Disease Control and Prevention (CDC) and the UK National Laboratories. Five years ago, the US National Institutes for Health (NIH) established the Vitamin D Standardization Program (VDSP) in an attempt to standardise the laboratory measurement of vitamin D across the globe, with key bodies signing up to it, including laboratories in the UK. In addition to this, there is now greater prominence than ever for the Vitamin D External Quality Assessment Scheme (DEQAS), which has been led by the UK. The DEQAS Advisory Panel performance targets call for 75% or more of the results falling within ± 25% of the Target Value (the National Institute of Standards and Technology (NIST) LC-MS/MS assigned value). The international DEQAS has been monitoring the performance of 25OHD assays since 1989 and now has >1000 registered participants worldwide. In essence, DEQAS is an ongoing multicentre trial of the methods used by its participants and provides a unique opportunity to assess the accuracy and specificity of 25OHD methods, as well as the analytical performance of a large number of their users. These initiatives are extremely important and can only serve to be beneficial to the vitamin D field in the long-term.

Low vitamin D status has been reported throughout the world – and there is, at last, recognition that this is a real problem in the 21st century. In the recent report by the International Osteoporosis Foundation (Mitchell et al. 2015), vitamin D insufficiency (25OHD status <50 nmol/l) has been identified as being prevalent in women of child-bearing age, pregnant women (see Toher et al. 2013), children and adolescents, as well as adults in general. Adults who are at particular risk of having low vitamin D status include individuals living at higher latitudes, such as in the UK and many parts of mainland Europe, with minimal exposure to sunlight; those who are overweight and obese; individuals with a darker skin tone; those who cannot expose their skin to the sun for medical or cultural reasons and populations who are institutionalised and spend very little time outdoors. Furthermore, individuals with diseases that reduce the uptake of vitamin D from the intestine are at an increased risk of low vitamin D status and require a special focus.

For the UK, vitamin D deficiency (as defined as a 25OHD status <25 nmol/l) is a major public health problem, particularly in older people (>65 years) and in the UK South Asian population (including children). In the largest longitudinal study available in South Asian women, funded by the Food Standards Agency, 25OHD status was below 25 nmol/l for the entirety of the year in both pre-menopausal and post-menopausal groups (Macdonald et al. 2011; Darling et al. 2013). The UK National Diet and Nutrition Survey (NDNS) shows there to be a high prevalence of low vitamin D status in adolescents, particularly boys (see Prentice 2013). Data from the NDNS in older children show that 19.7% of boys and 24.4% of girls aged 11–18 years had a vitamin D status below 25 nmol/l (Bates et al. 2014). It is of course well established that vitamin D deficiency is an issue in the growing child, particularly with respect to impairment of bone development and a reduced peak bone mass attainment. Severe vitamin D deficiency results in rickets in children (osteomalacia in adults) and must be avoided at all costs. In 2012, the Chief Medical Officer (CMO) for England, Professor Dame Sally Davies, called for a review of cost-effectiveness of making the Healthy Start programme universal and offering free vitamins to all children under the age of 5 years and this is currently under review by the National Institute for Health and Care Excellence (NICE) (Alderton 2014). Vitamin D deficiency in our ageing population is undeniably a huge problem and one which must be the target of focused public health attention in the future (Buttriss 2015).

Vitamin D has many functions in addition to its key role in the regulation of calcium and phosphorus homoeostasis. The active hormone, 1,25-dihydroxyvitamin D, binds to the vitamin D receptor (VDR) in a large number of cells to promote/suppress gene transcription and thus regulate cell function. The VDR is not just present in bone but in muscle, adipose tissue, immune systems, the central nervous and endocrine systems, and some cancer cells.

The evidence for the role of vitamin D in skeletal health is robust but what we lack are strong RCTs or prospective studies to establish whether there is a role for vitamin D in relation to other health outcomes. Certainly, vitamin D plays a key role in muscle health, particularly in older adults. By helping to maintain muscle function and prevent falling and reducing the risk of falling, this will undoubtedly be of benefit to our (growing) ageing population (McCarthy & Kiely 2015). There are important RCT studies underway for other health outcomes, and we await their completion and publication with anticipa-
Dietary vitamin D intake varies considerably worldwide and is directly associated with the extent of vitamin D fortification specific to the country. Vitamin D as cholecalciferol (vitamin D₃) and ergocalciferol (vitamin D₂) is legally permitted to be added to foods [only in cholecalciferol and ergocalciferol form as stated in Annex 1 of Regulation (EC) No 1925/2006, amended by the Commission Regulation (EC) No. 1170/2009]. In the UK, dietary intakes of vitamin D are low, typically varying between 100 and 200 IU (2.5–5 µg) per day across different population groups. The recent update of the vitamin D content of fortified foods and supplements in the UK NDNS Nutrient Databank is very important and will ensure that dietary intake estimates of vitamin D in the UK are accurate and informative (Allen et al. 2014).

Vitamin D is naturally present in foods in two forms, with vitamin D₂ being present in plants and fungi whereas vitamin D₃ is found in fish, meat and eggs. There has been controversy for many years as to whether it matters if vitamin D₂ or vitamin D₃ is used as the source for raising vitamin D status (either as supplements or through food fortification) (see Tripkovic 2013). Results from the D₂-D₃ study, which has been funded by the Biotechnology and Biological Sciences Research Council (BBSRC) – Diet and Health Research Industry Club (DRINC) programme and is the largest RCT to directly compare vitamin D₂ with vitamin D₃, has shown some exciting results in both Caucasian and South Asian populations (Wilson et al. 2015) and there are intriguing new leads on mechanisms of action (Professor C. P. Smith & L. R. Wilson, personal communication 2015).

It is important to note that there are currently very few foods in the UK providing a valuable contribution to vitamin D intake, suggesting that food fortification with vitamin D could be an effective way of improving vitamin D status. This is a topic that requires urgent consideration from the consumer perspective – which foods would be most effective; and from a food industry perspective – to which foods can vitamin D be added in appropriate amounts.

The setting of dietary requirements for vitamin D has proved just as controversial as defining the level of 25OHD status to signify vitamin D ‘sufficiency’ (see Cashman & Kiely 2014). The Scientific Advisory Committee on Nutrition’s Vitamin D and Health draft report released for consultation in July 2015 is a landmark for the vitamin D field in the UK (see Buttriss 2015). Whilst there are more questions than answers (as is the case for so many nutrients), a new era of vitamin D exploration is upon us – the future will be exciting, challenging and revealing!

S. A. Lanham-New
Head, Nutritional Sciences Department, School of Biosciences and Medicine, University of Surrey, Guildford, Surrey, UK

L. R. Wilson
BBSRC DRINC PhD Research Fellow, Nutritional Sciences Department, School of Biosciences and Medicine, University of Surrey, Guildford, Surrey, UK

References

McCarthy EK & Kiely M (2015) Vitamin D and muscle strength throughout the life course: A review of epidemiological and inter-

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Dear <Name>

The Practice has been made aware of a new research study at the University of Surrey in Guildford, investigating how much Vitamin D is needed in the diet everyday to be healthy.

This research has been reviewed by a committee on Human ethics (South East Coast – Surrey Research Ethics Committee) and the University of Surrey Ethics Committee and is funded by the Biotechnology and Biological Sciences Research Council, a government-funded organisation that supports health-related research in the UK.

We would like to give as many of our patients as possible an opportunity to participate in medical research.

Therefore the research team would like to hear from women who are healthy, of Caucasian or South Asian origin, aged 20-64 years old and who are interested in participating in this study. The study would involve attending the University of Surrey on three occasions over 12 weeks and being prepared to consume specially made food and drink items over that time (a summary information sheet about the study is enclosed with this letter). Travel expenses for the use of public transport or private car will be reimbursed upon provision of a valid receipt or evidence of mileage.

If you would like further details, please contact the research team directly:

Dr Laura Tripkovic, Registered Dietician
Nutritional Sciences Division.
Tel: 01483 689222
Email: d2d3@surrey.ac.uk

Occasionally our computer database search incorrectly identifies people who would be suitable for the research study. If this is the case then please accept our apologies for this inconvenience. I would like to assure you that your patient confidentiality has in no way been compromised and at no time have any of the researchers had access to your personal information.

Yours sincerely,

Dr ……………..

Dr …………….. on behalf of ……………..Practice
You are invited to take part in a Vitamin D nutrition research study based at the University of Surrey based in Guildford. Below is a summary of the study including information about why the study is taking place and an outline of what would be involved for you if you chose to take part. Please take your time to read this document carefully and if you would like further information about this study then please contact Dr Laura Tripkovic, email: d2d3@surrey.ac.uk or tel: 01483 689222. Thank you for your time and attention.

The purpose of the D2-D3 Study
Vitamin D is an important part of our diet as it helps to keep bones and muscles strong and healthy. Some foods in our diet (such as eggs, oily fish and fortified breakfast cereals and margarines) can give us small amounts of vitamin D. We are also able to make vitamin D in our skin when we spend some time out in the sun during the months of April to September. Yet research from the University of Surrey has shown that the vitamin D in our diet and the amount we get from the sun is usually not enough to keep our levels of vitamin D healthy during the winter months, which may cause problems for future bone health. Therefore the D2-D3 Study aims to investigate whether consuming food and drink items with a specific amount of added vitamin D in, is enough to keep blood levels of vitamin D within the healthy range during the winter months.

Who can participate in the study?
We are looking to recruit a total of 355 women for this study and we would like to hear from any lady who is of Caucasian or South Asian origin, aged 20-64 years, has a body mass index of 18-30 kg/m² and is in good health.

What is involved in the study?
The main commitment of the study is to consume a juice and biscuit everyday for 12 weeks. One product will be a placebo, the other may contain vitamin D or it may not, whether the second product contains vitamin D or not will be chosen completely at random. To enter this study you must be prepared to consume the products that you have been assigned without knowing whether they contain vitamin D.

There are three visits to the University of Surrey – at the beginning, middle and end of the study. All visits must take place in the morning before breakfast and a breakfast will be provided after the visit, as will an opportunity to claim for travel expenses.

At each study visit, a small blood sample will be taken (about the same as 5 teaspoons), as well as blood pressure, weight and waist measurements. At the beginning and end of the study, a diet diary is completed for four days and a dosimeter badge to measure sunlight exposure would be worn for seven days.

At the first visit only, a bone scan will be conducted to determine bone strength and your risk of osteoporosis.

What are the possible benefits to taking part in this study?
The main benefit of taking part in this study (if assigned to the vitamin D products) will be the consistent supplementation of Vitamin D through the winter months where Vitamin D availability is dramatically reduced due to the weak sunlight. In addition, the information that you provide in this study will be of value to science with respect to whether the UK should have a dietary recommendation for vitamin D. You will receive information on your diet, as well as the results of your bone health measurements and your vitamin D status.
# Health and Lifestyle Questionnaire

Ergocalciferol (Vitamin D₂) vs. Cholecalciferol (Vitamin D₃) Food Fortification: Comparative Efficiency in raising 25OHD Status in Asian & Caucasian Women and Mechanisms of Action (D2-D3 Study)

**Screening Number:**

**Ethnicity:**

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<td>Irish</td>
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<td>Any other White background (please state)</td>
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<td>Indian</td>
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<td>Pakistani</td>
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**Partly-veiled? (i.e. Hijab or headscarf)**

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**Height:**

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<tr>
<th>BMI kg/m²</th>
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**Pre-menopausal: YES/NO**

**Post-menopausal (incl years post-menopause) YES/NO**

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<th>_____yrs</th>
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</thead>
</table>
Tick all that apply:

| Prior/present history of coronary heart disease, angina, heart attack or stroke |
| Prior/present history of Type 1 and Type 2 Diabetes. |
| Prior/present history of Thyroid disease |
| Prior/present history of osteoporosis, osteopenia or other musculoskeletal disease |
| Prior/present history of haematological disease (except mild anaemia) |
| Prior/present history of malignancy |
| Prior/present history of a gastrointestinal disorder, such as Crohns Disease, Coeliac Disease or Irritable Bowel Syndrome. |
| Prior/present history of liver or kidney disease. |
| Prior/present history of clinical depression or other psychological disorders. |
| Prior/present history of eating disorders. |
| Prior/present history of drug or alcohol abuse within the last 2 years. |

Are you on any regular medication prescribed by your GP or other health-care provider?  YES/NO
If yes, please state which type and how often you take them

Do you take any dietary supplements such as vitamins, minerals or fish oils?  YES/NO
If yes, please state which type and how often you take them.

Are you currently on a weight-reducing diet or other dietary restrictions?  YES/NO
If yes, please give details. Also please include details of food intolerances and allergies.
Have you visited any hot/sunny countries in the last month? YES/NO
If yes, please state the country and length of stay.

Do you plan on visiting a hot/sunny country in the next 6 months? YES/NO
If yes, please state the country and length of stay.

Do you regularly use sunbeds? YES/NO
If yes, please state frequency and duration of use.

Are you pregnant or planning a pregnancy within the next six months? YES/NO

Are you breast-feeding? YES/NO

Have you been involved in a clinical trial in the last 4 months? YES/NO
If yes, please give details.
Do you smoke? YES/NO
If yes, how many per day?

Do you drink alcohol? YES/NO
If yes, how many units per week? (See guide below)

End of questionnaire

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<tr>
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<th>Unit</th>
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<tr>
<td>Ordinary strength lager (4%) e.g. Carling, Fosters</td>
<td>Pint</td>
<td>2.3</td>
</tr>
<tr>
<td>Strong lager (5.2%) e.g. Stella Artois, Kronenburg</td>
<td>Pint</td>
<td>3.0</td>
</tr>
<tr>
<td>Strong lager e.g. Stella Artois, Carlsberg Export, Grolsch</td>
<td>440ml can</td>
<td>2.2</td>
</tr>
<tr>
<td>Beer/ordinary strength Ale e.g. John Smith’s, Guinness</td>
<td>Pint</td>
<td>2.3</td>
</tr>
<tr>
<td>Red/White Wine</td>
<td>Std 175ml</td>
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<td>Red/White Wine</td>
<td>Lg. 250ml</td>
<td>3.0</td>
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<td>Spirits</td>
<td>Lg. 35ml</td>
<td>1.4</td>
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</table>

What is a unit of Alcohol?

The list below shows the approximate number of units of alcohol in common drinks:-
13 July 2011

Prof. Susan Lanham-New
Head of Division: Nutritional Sciences
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH

Dear Prof. Lanham-New

Study title: Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

REC reference: 11/LO/0708
Protocol number: D2-D3/0311

Thank you for your letter of 08 July 2011, responding to the Committee’s request for further information on the above research.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting revised documents, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see “Conditions of the favourable opinion” below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.
Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdukforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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</thead>
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<tr>
<td>Advertisement</td>
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<td>04 May 2011</td>
</tr>
<tr>
<td>Covering Letter</td>
<td></td>
<td>05 July 2011</td>
</tr>
<tr>
<td>Evidence of insurance or indemnity</td>
<td></td>
<td>05 July 2010</td>
</tr>
<tr>
<td>GP/Consultant Information Sheets</td>
<td>1</td>
<td>30 April 2011</td>
</tr>
<tr>
<td>Investigator CV</td>
<td></td>
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<tr>
<td>Letter from Sponsor</td>
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<td>12 May 2011</td>
</tr>
<tr>
<td>Letter of invitation to participant</td>
<td>1.1</td>
<td>21 June 2011</td>
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<tr>
<td>Other: Letter of Invitation - Recruit Via advertisement</td>
<td>1</td>
<td>30 April 2011</td>
</tr>
<tr>
<td>Other: Food Diary</td>
<td></td>
<td>Undated</td>
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<tr>
<td>Other: Letter from Funder</td>
<td></td>
<td>02 September 2010</td>
</tr>
<tr>
<td>Other: CV - Dr Jacqueline Berry</td>
<td></td>
<td>Undated</td>
</tr>
<tr>
<td>Other: CV - Dr Kathryn Hart</td>
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<td>Undated</td>
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<td>Other: CV - Dr Elina Hypponen</td>
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<td>Undated</td>
</tr>
<tr>
<td>Other: CV - Dr Simon Penson</td>
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<tr>
<td>Other: CV - Prof Colin Smith</td>
<td></td>
<td>Undated</td>
</tr>
<tr>
<td>Other: CV - Dr Laura Tripkovic</td>
<td></td>
<td>Undated</td>
</tr>
<tr>
<td>Other: Subject Screening &amp; Enrolment Log</td>
<td>1</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Other: Confidential Personal Information Form</td>
<td>1</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Participant Consent Form: Main Study</td>
<td>1.4</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Participant Consent Form: Sub-Study</td>
<td>1.2</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Participant Information Sheet: Main Study</td>
<td>1.4</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Participant Information Sheet: Sub-Study</td>
<td>1.2</td>
<td>21 June 2011</td>
</tr>
<tr>
<td>Protocol</td>
<td>1.4</td>
<td>05 May 2011</td>
</tr>
<tr>
<td>Questionnaire: Adverse Event and Compliance Interview</td>
<td>1</td>
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<td>Questionnaire: Health and Lifestyle</td>
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<td>REC application</td>
<td>3.1</td>
<td>14 May 2011</td>
</tr>
</tbody>
</table>
Response to Request for Further Information | 08 July 2011
Summary/Synopsis | 05 May 2011

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

Further information is available at National Research Ethics Service website > After Review

11/LO/0708 Please quote this number on all correspondence

With the Committee’s best wishes for the success of this project

Yours sincerely

Prof David Russell-Jones
Chair

Email: rsc-tr.ethicscommittee@nhs.net

Enclosures: “After ethical review – guidance for researchers”

Copy to: Dr Laura Tripkovic – email – laura.tripkovic@surrey.ac.uk
University of Surrey

Glenn Moulton – email – g.moulton@surrey.ac.uk
University of Surrey

Helen Evans – email – helen.evans@wsht.nhs.uk
Sussex NHS Research Consortium
02 August 2011

Prof Susan Lanham-New
Head of Division: Nutritional Sciences
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH

Dear Prof Lanham-New

Study title: Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

REC reference: 11/LO/0708
Protocol number: D2-D3/0311
Amendment number: 1
Amendment date: 26 July 2011

The above amendment was reviewed at the meeting of the Sub-Committee held on 02 August 2011.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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</tbody>
</table>

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D
approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/LO/0708: Please quote this number on all correspondence

Yours sincerely

Prof David Russell-Jones
Chair

E-mail: rsc-tr.ethicscommittee@nhs.net

<table>
<thead>
<tr>
<th>Enclosures:</th>
<th>List of names and professions of members who took part in the review</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Copy to:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Laura Tripkovic – email – <a href="mailto:laura.tripkovic@surrey.ac.uk">laura.tripkovic@surrey.ac.uk</a></td>
<td>University of Surrey</td>
</tr>
<tr>
<td>Helen Evans – email – <a href="mailto:helen.evans@wsht.nhs.uk">helen.evans@wsht.nhs.uk</a></td>
<td>Sussex NHS Research Consortium</td>
</tr>
</tbody>
</table>
15 September 2011

Prof. Susan Lanham-New  
Head of Division: Nutritional Sciences  
Faculty of Health and Medical Sciences  
University of Surrey  
Guildford, Surrey  
GU2 7XH

Dear Prof. Lanham-New

Study title: Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

REC reference: 11/LO/0708  
Protocol number: D2-D3/0311  
Amendment number: 3.1  
Amendment date: 05 September 2011

The above amendment was reviewed at the meeting of the Sub-Committee held on 14 September 2011.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.
**R&D approval**

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/LO/0708: Please quote this number on all correspondence

Yours sincerely

Prof David Russell-Jones  
Chair

E-mail: rsc-tr.ethicscommittee@nhs.net

<table>
<thead>
<tr>
<th>Enclosures:</th>
<th>List of names and professions of members who took part in the review</th>
</tr>
</thead>
</table>

| Copy to: | Dr Laura Tripkovic – email – laura.tripkovic@surrey.ac.uk  
University of Surrey  
Helen Evans – email – Helen.evans@wsht.nhs.uk  
Sussex NHS Research Consortium |
|-----------|-----------------------------------------------------------------|
02 November 2011

Prof. Susan Lanham-New
Head of Division: Nutritional Sciences
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH

Dear Prof. Lanham-New

Study title: Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

REC reference: 11/LO/0708
Protocol number: D2-D3/0311
Amendment number: 1.2
Amendment date: 20 October 2011

The above amendment was reviewed at the meeting of the Sub-Committee held on 20 October 2011 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

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<tr>
<th>Document</th>
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<td>Recruitment Poster</td>
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<td>adverse event and compliance interview</td>
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<td>19 October 2011</td>
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<td>GP/Consultant Information Sheets</td>
<td>1.2</td>
<td>19 October 2011</td>
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<td>Participant Consent Form: Main study</td>
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<td>Participant Information Sheet: Main study</td>
<td>1.6</td>
<td>19 October 2011</td>
</tr>
<tr>
<td>Protocol</td>
<td>1.6</td>
<td>19 October 2011</td>
</tr>
</tbody>
</table>
Notice of Substantial Amendment (non-CTIMPs) 1.2 20 October 2011

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/LO/0708: Please quote this number on all correspondence

Yours sincerely

Prof David Russell-Jones
Chair

E-mail: georgina.marshall@nhs.net

Enclosures: List of names and professions of members who took part in the review

Copy to: Glenn Moulton, University of Surrey
Helen Evans, Sussex NHS Research Consortium
22 June 2012

Prof. Susan Lanham-New
Head of Division: Nutritional Sciences
University of Surrey
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH

Dear Prof. Lanham-New

Study title: Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

REC reference: 11/LO/0708
Protocol number: D2-D3/0311
Amendment number: 3.4
Amendment date: 09 May 2012

The above amendment was reviewed at the meeting of the Sub-Committee held on 25 May 2012 by the Sub-Committee in correspondence.

Ethical opinion

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

<table>
<thead>
<tr>
<th>Document</th>
<th>Version</th>
<th>Date</th>
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<tr>
<td>Search Terms</td>
<td>1.0</td>
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<tr>
<td>Summary Participant Information Sheet</td>
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<td>16 April 2012</td>
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<td>Recruitment participants via GP Letter</td>
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<td>16 April 2012</td>
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<tr>
<td>Participant Consent Form: Sub-Study Consent Form</td>
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<tr>
<td>Participant Information Sheet: Sub Study Participant Information</td>
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<td>08 May 2012</td>
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</table>
Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/LO/0708: Please quote this number on all correspondence

Yours sincerely

[Signature]

Professor David Russell-Jones
Chair

E-mail: georgina.marshall@imperial.nhs.uk

Enclosures: List of names and professions of members who took part in the review

Copy to: Helen Evans, Sussex NHS Research Consortium
Dr Laura Tripkovic
Professor Susan Lanham-New  
Nutritional Sciences  
FHMS

Ehics Committee

22 August 2011

Dear Professor Lanham-New

**Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study) Fast-Track EC/2011/97/FHMS**

On behalf of the Ethics Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the submitted protocol and supporting documentation.

Date of confirmation of ethical opinion: 22 August 2011.

The list of documents reviewed and approved by the Committee under its Fast Track procedure is as follows:-

<table>
<thead>
<tr>
<th>Document</th>
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<tbody>
<tr>
<td>Summary of the project</td>
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<tr>
<td>Research Protocol</td>
</tr>
<tr>
<td>Confirmation of acceptance of a BBSRC grant</td>
</tr>
<tr>
<td>Main study Participant Information sheet</td>
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<tr>
<td>Sub-Study Participant Information sheet</td>
</tr>
<tr>
<td>D2-D3 Study Consent form</td>
</tr>
<tr>
<td>D2-D3 Study Consent form (Sub-Study)</td>
</tr>
<tr>
<td>Health and Lifestyle Questionnaire</td>
</tr>
<tr>
<td>Adverse Event and compliance interview – visits 2 &amp; 3</td>
</tr>
<tr>
<td>D2 –D3 Study: Adverse Events Log</td>
</tr>
<tr>
<td>D2-D3 Study: Confidential Personal Information Form</td>
</tr>
<tr>
<td>D2-D3 Study Subject Screening and Enrolment Log</td>
</tr>
<tr>
<td>Standard letters (3)</td>
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<tr>
<td>Risk assessment</td>
</tr>
<tr>
<td>Protocol Submission Proforma: Insurance</td>
</tr>
<tr>
<td>Sponsor letter (GM)</td>
</tr>
<tr>
<td>Recruitment advert</td>
</tr>
<tr>
<td>Confirmation of favourable ethical opinion from NRES Committee South East Coast - Surrey</td>
</tr>
</tbody>
</table>

This opinion is given on the understanding that you will comply with the University’s Ethical Guidelines for Teaching and Research.

The Committee should be notified of any amendments to the protocol, any adverse reactions suffered by research participants, and if the study is terminated earlier than expected with reasons. Please be advised that the Ethics Committee is able to audit research to ensure that researchers are abiding by the University requirements and guidelines.
You are asked to note that a further submission to the Ethics Committee will be required in the event that the study is not completed within five years of the above date.

Please inform me when the research has been completed.

Yours sincerely

[Signature]

Glenn Moulton
Secretary, University Ethics Committee
Registry

cc: Professor S Williamson, Chairman, Ethics Committee
Dr Laura Tripkovic  
Nutritional Sciences  
FHMS

02 September 2011

Dear Dr Tripkovic

Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study) Fast-Track EC/2011/97/FHMS

I am writing to inform you that the Chairman, on behalf of the Ethics Committee, has considered the Amendments requested to the above protocol and has approved them on the understanding that the Ethical Guidelines for Teaching and Research are observed. Please be advised that the Ethics Committee is able to audit research to ensure that researchers are abiding by the University requirements and guidelines.

If the project includes distribution of a survey or questionnaire to members of the University community, researchers are asked to include a statement advising that the project has been reviewed by the University’s Ethics Committee.

Date of confirmation of ethical opinion: 22 August 2011.

Date of favourable ethical opinion of amendment to protocol: 2 September 2011.

The list of amended documents reviewed and approved by the Chairman is as follows:-

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<tr>
<th>Document</th>
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<tr>
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<td>Recruitment poster</td>
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<tr>
<td>Recruit participant via GP letter</td>
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</tbody>
</table>

Yours sincerely

Glenn Moulton  
Secretary, University Ethics Committee  
Registry

cc: Professor S Williamson, Chairman, Ethics Committee
Professor Susan Lanham-New  
Nutritional Sciences Division  
FHMS  

08 November 2011  

Dear Professor Lanham-New  

Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification:  
Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and  
Mechanisms of Action (D2-D3 Study) Fast-Track EC/2011/97/FHMS  

I am writing to inform you that the Chairman, on behalf of the Ethics Committee, has  
considered the Amendments requested to the above protocol and has approved them  
on the understanding that the Ethical Guidelines for Teaching and Research are  
observed. Please be advised that the Ethics Committee is able to audit research to  
ensure that researchers are abiding by the University requirements and guidelines.  

If the project includes distribution of a survey or questionnaire to members of the  
University community, researchers are asked to include a statement advising that the  
project has been reviewed by the University's Ethics Committee.  

Date of confirmation of ethical opinion: 21 August 2011.  

Date of favourable ethical opinion of amendment to protocol: 8 November 2011  

The list of amended documents reviewed and approved by the Chairman is as follows:- 

<table>
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<tr>
<th>Document</th>
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<tr>
<td>Detailed protocol</td>
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<td>Participant Information sheet</td>
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<tr>
<td>Consent form</td>
</tr>
<tr>
<td>NHS REC confirmation of favourable ethical opinion for amendment</td>
</tr>
<tr>
<td>Recruit participants via GP letter</td>
</tr>
<tr>
<td>GP/Consultant Information sheet</td>
</tr>
<tr>
<td>Recruitment advert</td>
</tr>
<tr>
<td>Adverse Event and Compliance interview – visits 2 &amp; 3</td>
</tr>
<tr>
<td>D2 – D3 Study – Adverse Events Log</td>
</tr>
</tbody>
</table>

Yours sincerely  

Glenn Moulton  
Secretary, University Ethics Committee Registry  

cc: Professor S Williamson, Chairman, Ethics Committee
22 June 2012

Prof. Susan Lanham-New
Head of Division: Nutritional Sciences
University of Surrey
Faculty of Health and Medical Sciences
University of Surrey
Guildford, Surrey
GU2 7XH

Dear Prof. Lanham-New

**Study title:** Ergocalciferol (Vitamin D2) vs. Cholecalciferol (Vitamin D3) Food Fortification: Comparative Efficiency in raising 25OHD Status in Caucasian & Asian Women and Mechanisms of Action (D2-D3 Study)

**REC reference:** 11/LO/0708
**Protocol number:** D2-D3/0311
**Amendment number:** 3.4
**Amendment date:** 09 May 2012

The above amendment was reviewed at the meeting of the Sub-Committee held on 25 May 2012 by the Sub-Committee in correspondence.

**Ethical opinion**

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

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<thead>
<tr>
<th>Document</th>
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<th>Date</th>
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<td>Recruitment participants via GP Letter</td>
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<td>16 April 2012</td>
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<tr>
<td>Participant Consent Form: Sub-Study Consent Form</td>
<td>1.4</td>
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<tr>
<td>Participant Information Sheet: Sub Study Participant Information</td>
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<td>08 May 2012</td>
</tr>
</tbody>
</table>
Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

11/LO/0708: Please quote this number on all correspondence

Yours sincerely

[Signature]

Professor David Russell-Jones
Chair

E-mail: georgina.marshall@imperial.nhs.uk

Enclosures: List of names and professions of members who took part in the review

Copy to: Helen Evans, Sussex NHS Research Consortium
Dr Laura Tripkovic
PROTOCOL TITLE:
Ergocalciferol (Vitamin D₂) vs. Cholecalciferol (Vitamin D₃) Food Fortification: Comparative Efficiency in raising 25OHD Status in Asian & Caucasian Women and Mechanisms of Action (D2-D3 Study)

Trial Identifiers
Study Protocol: D2-D3/0311
BBSRC DRINC: BB/I006192/1
University of Surrey REC: EC/2011/97/FHMS
South-East Coast (NHS) REC: 11/LO/0708
NHS R&D: CSP 78820
ISRCTN: ISRCTN23421591

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1. Background & Rationale

Vitamin D is the term used to describe two molecules, ergocalciferol (Vitamin D$_2$) and cholecalciferol (Vitamin D$_3$). The former is derived by ultra-violet irradiation of the ergosterol that is widely distributed in plants and fungi whereas cholecalciferol is formed from the action of ultra-violet irradiation on the skin. It is known that poor vitamin D status is a very common problem in the UK. It is also known that a lack of vitamin D availability has potentially serious health implications, including links to issues with muscle/bone function and increasing the risk of diabetes. With the combination of poor vitamin status and the potential risks to health, it is clear that finding ways of improving vitamin D intake that are acceptable as a public health strategy are urgently needed.

Research that has just been completed by the Nutritional Sciences Division (PI: Prof Susan Lanham-New) at the University of Surrey (Food Standards Agency funding; Project No. NO5064; £0.5M) indicated the following:

- Within the UK population dietary intake of vitamin D is too low to have any effect on vitamin D status as there are too few foods providing a valuable natural source
- South Asian women are extremely vitamin D deficient
- Caucasians have extensive vitamin D insufficiency

There is evidence within the literature of differences in key polymorphisms of important genes that are critical to vitamin D metabolism in Asian Indian vs. Caucasian as well as differences in key vitamin D metabolism enzymes. This requires a fuller investigation since there are differences in the availability of vitamin D$_2$ and vitamin D$_3$, and hence public health advice on increasing vitamin D intake (particularly with respect to the development of vitamin D-rich food products) needs to confirm that either form of vitamin D is effective. Furthermore, the South Asian community are vegan/strict vegetarians (~26%) and hence the source of vitamin D$_3$ is a problem in this group due to it being derived from animals (vitamin D$_3$ supplements come from Lanolin, which is extracted from sheep’s wool.)

In addition to the need for clarification regarding the optimal level of daily vitamin D intake, there is currently controversy as to the effectiveness of vitamin D$_2$ and vitamin D$_3$ respectively in raising 25OHD (a marker of vitamin D levels in blood). The most recently published data suggests that D$_2$ and D$_3$ are equally effective which contradicts previous thinking; therefore this issue of ambiguity clearly requires urgent attention and will be addressed within the D2-D3 Study.

There are currently no intervention studies that have comprehensively investigated the issues surrounding vitamin D as described above. The proposed D2-D3 Study will enable a better understanding of how comparable the two forms of vitamin D are at raising vitamin D status in Caucasians and Asians, and investigate the mechanisms of action with respect to any differences observed between the two vitamin D forms or between ethnic groups. Mechanisms of action will focus on genetic differences as well as differences in vitamin D metabolising enzymes.

The results obtained from this significant study will not only inform the scientific community but also be a critical resource for key stakeholders (i.e. food industry, government health agencies) to collaborate in determining future public health strategies and thus potentially positively impacting on the health of the population for years to come.

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2. Trial Objectives & Design

2.1 Primary Trial Objectives:

To compare the efficiency of food and drink fortification with 15µg/d [600 IU/d] of ergocalciferol (Vitamin D$_2$) vs. cholecalciferol (Vitamin D$_3$) in raising 25OHD levels in Asian and Caucasian women.

2.2 Secondary Trial Objectives

(i) To determine which food vehicle for fortification (i.e. a SOLID vs. LIQUID food) with Vitamin D$_2$ vs. Vitamin D$_3$ is more effective in raising 25OHD levels, independent of ethnicity.

(ii) To investigate whether 15µg/d [600 IU/d] is effective in raising wintertime 25OHD levels above ‘deficiency/insufficiency’ thresholds (25nmol/l and 40nmol/l respectively) in Caucasian and Asian women and whether there are any differences in the effects of Vitamin D$_2$ vs. Vitamin D$_3$ fortification, independent of ethnicity.

(iii) To investigate the mechanisms (genetic/enzymatic) underlying the differences observed in the preceding objectives via genotyping the participants for polymorphisms related to vitamin D metabolism. These will include known candidate variants (VDR, vitamin D binding protein and CYP27A1), as well as variants identified by the ongoing genome-wide association analyses on 25OHD. This will enable a genetic risk score to be developed, based on the vitamin D associated variants, and thus characterise differences in genetic influences on vitamin D metabolism between Caucasian and South Asian women. We propose to store the DNA samples after the above analysis so that they may potentially be analysed in greater detail in the future, by more comprehensive emerging techniques. Prior to any such analysis participants will be contacted and additional ethical approval will be sought.

(iv) To compare the effectiveness of Vitamin D$_2$ vs. Vitamin D$_3$ in raising 25OHD levels above deficiency/insufficiency and sufficiency cut-offs in Asian and Caucasian women in the two types of intervention products. Secondary analyses will include evaluation of variations in the response to fortification by single SNP variations and by the genetic vitamin D risk score. Main comparisons will be carried out on women classified in the top and bottom quartiles, with further analysis focussing on effect modification in vitamin D influence on the 25OHD concentration by genetic risk.

(v) To determine, through transcriptomic (global mRNA) analysis of leukocyte samples, genome-wide differences in gene expression, in the Caucasian and South Asian women in response to the Vitamin D$_2$ and Vitamin D$_3$ fortification. This analysis will also focus on comparing the best and worst fortification responders in each group (accounting for ethnic origin) to enable us to undertake a comparison of the best and worst responders in terms of their gene expression. If new techniques become available in the future that enable us to analyse the gene expression in further detail, then participants will be contacted and additional ethical approval will be sought.

(vi) To examine the effects of vitamin D$_2$ and D$_3$ on DNA repair activity in peripheral blood mononuclear cells in a subset of participants. This analysis will be included as a recent in vitro study has provided evidence that vitamin D can influence DNA repair processes (Suarez-Gonzalez et al. *Embo J* 2011, 30: 3383-96). We will make use of novel assays for determining DNA repair capacity in cell extracts, currently in development within the Faculty D2-D3 Study Protocol Version 1.7 (08/05/2012)

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of Health & Medical Sciences at Surrey, to examine the effects in human subjects. Moreover, the gene expression analysis, described above, will provide scope to examine the relationships between repair activities and patterns of DNA repair gene expression at the level of the gene transcripts.

2.3 Hypothesis:

We propose that ergocalciferol (Vitamin D2) and cholecalciferol (Vitamin D3) will not be equally efficacious in raising 25OHD status; that Asian and Caucasian women will show a significantly different response to dietary fortification with both Vitamin D2 and D3. We predict that some of these differences will be reflected in identifiable differences in leukocyte-derived gene expression profiles.

2.4 Trial Design

The D2-D3 Study is a food fortification trial and will be a randomised double-blind, controlled parallel design. Participants will consume both a biscuit and a fruit juice (“intervention products”) daily that will be enriched in Vitamin D2, Vitamin D3 or is a placebo for 12 weeks. Participants will be assessed at baseline, week 6 and week 12 of the trial (see figure below.)

Trial Flowchart

Screening
Informed consent
Medical history & Screening bloods
Check inclusion/exclusion criteria

Randomisation
Caucasian n=265, S.Asian n=90

Group A
BISCUIT: 600IU/15µg Ergocalciferol (Vitamin D2)
JUICE: Placebo
Caucasian n=53, S.Asian n=18

Group B
JUICE: 600IU/15µg Ergocalciferol (Vitamin D2)
BISCUIT: Placebo
Caucasian n=53, S.Asian n=18

Group C
BISCUIT: 600IU/15µg Cholecalciferol (Vitamin D3)
JUICE: Placebo
Caucasian n=53, S.Asian n=18

Group D
JUICE: 600IU/15µg Cholecalciferol (Vitamin D3)
BISCUIT: Placebo
Caucasian n=53, S.Asian n=18

Group E
JUICE & BISCUIT: Placebo
Caucasian n=53, S.Asian n=18
2.5 Sample size

A total of 320 subjects (at 90% power) are required for recruitment into the five key intervention groups. This has been calculated as follows: 240 Caucasian women ($n = 48$ in each of the five groups) and 80 partly-veiled Pakistani/Indian/Bangladeshi/Arabian women ($n = 16$ in each group). These will be randomly recruited to one of five intervention groups (‘SOLID’ Vit D$_2$ with LIQUID placebo (Group A) vs. SOLID Vit D$_3$ with LIQUID placebo (Group C), vs. SOLID Placebo with LIQUID placebo (Group E) vs. LIQUID Vit D$_2$ with SOLID placebo (Group B) vs. LIQUID Vit D$_3$ with SOLID placebo (Group D). Using data from published research papers [1-4], we calculate this will enable us to: (i) detect a 0.6 SD size effect at 90% power in serum 25OHD levels between placebo and 600IU in Caucasian women for Vitamin D$_2$ vs. Vitamin D$_3$; (ii) detect a 1.1 SD size effect at 90% power in plasma 25OHD levels between treatment groups in Asian for Vitamin D$_2$ vs. Vitamin D$_3$. We will over recruit to allow for ≈10% drop-out rates ($n = 35$) which will mean maximal recruitment of 265 Caucasian women and 90 partly-veiled women. These numbers will enable us to detect a 0.8SD size effect at 90% power in 25OHD levels between Asian and Caucasian women and a 0.7SD size effect at 90% power for a difference in 25OHD levels between the Vitamin D$_2$ vs. Vitamin D$_3$ groups.


2.6 Randomisation procedure

Participants will be randomised using a simple computer-generated randomisation programme. The randomisation will be stratified to take into account the participants’ ethnicity, BMI and age, and will be verified by the trial statistician with the codes assigned to the participants by a trial investigator (the investigator will be blinded to the randomisation). The trial statistician will be responsible for keeping the code.

The fortification of the biscuit and juice drink intervention products with either vitamin D$_2$, vitamin D$_3$ or the placebo will be blinded during the process of packaging. Un-blinding will only occur in the event of a health issue where the consumption of the intervention product is suspected to be the cause or wherever the un-blinding will be considered essential for the medical care of the participant.

2.7 Statistical analysis

Statistical analysis of the data will be undertaken with support from the University of Surrey statistical department and the UCL Institute of Child Health. Data will be checked for normality using appropriate testing. Analysis of the intervention data will be divided into two phases: Intention to Treat (ITT) and Analysis per Protocol and appropriate parametric/non-parametric analysis will be applied. Analyses will also include evaluation of variations in the response to fortification by single SNP variations and by the genetic vitamin D risk score.

Diet diary data will be analysed using WinDiets (dietary analysis programme), with standardised portion sizes obtained from the ‘Food Portion Sizes’ book (Food Standards
Agency). The data produced will assist in the assessment of participant compliance to the consumption of the intervention products, and also be analysed as a potential confounding factor during the final analysis.

3. Selection and withdrawal of participants

3.1 Selection of participants

Participants will be primarily recruited from the databases and networks that were established during the D-FINES trial (NHS REC No.O6/Q1090/1). Participants will also be recruited from the University of Surrey and the local population by poster and use of media advertisement (newspaper and radio) in addition to the research team visiting local businesses and community groups. A collaboration with the Primary Care Research Network (South-East) will enable recruitment of potential participants via GP practices within the Surrey Primary Care Trust. As part of the PCRN collaboration, potential participants identified within the GP practices (via specific and accurate searching of patient databases using criteria set in the document ‘Search Terms, version 1.0’), will receive a letter introducing them to the study, with a summary information sheet about the D2-D3 Study also enclosed.

At the baseline visit, prior to commencing any trial-related activities, participants will be taken through the process of informed consent for the trial by an investigator who is trained in the Human Tissue Act (2004) and the procedure of taking informed consent. All participants will have been given ample time (up to 2 weeks) to consider the details of the trial and ask any questions that they may have before deciding whether to participate in the trial or not.

All participants will be screened via the inclusion and exclusion criteria set out below in sections 3.2 and 3.3. Prospective participants will be asked to self-report their current health and lifestyle via telephone interview, and will be excluded based on the information volunteered by them and dependant on satisfactory screening blood test results (testing for anaemia, kidney, liver and thyroid function).

If participants are accepted on to the trial, their General Practitioner will be informed via letter. This practise will be made clear to the participant during the consenting procedure and is a condition of participation in the trial.

3.2 Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion</th>
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<tbody>
<tr>
<td><strong>Gender</strong></td>
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<td>Female</td>
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<tr>
<td><strong>Age</strong></td>
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<tr>
<td>20-64 years</td>
<td>aged 19 yrs and under</td>
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<td></td>
<td>aged 65 yrs and over</td>
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<tr>
<td><strong>Birth Month &amp; Year</strong></td>
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<tr>
<td>April 1948 to Nov 1992 inclusive</td>
<td>After Nov 1992</td>
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<td></td>
<td>Before April 1948</td>
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<tr>
<td><strong>Ethnicity</strong></td>
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<tr>
<td>White – British</td>
<td>Mixed Race</td>
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<tr>
<td>White – Irish</td>
<td>Black Caribbean</td>
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<tr>
<td>White – Any other white background</td>
<td>Black African</td>
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| South Asian/South Asian British – Indian | Black - other |
| South Asian/South Asian British – Pakistani | East Asia – |
| South Asian/ South Asian British – Bangladeshi | China/Japan/Korea/Malaysia/Philippines |
| Arab/Arab British – Kuwait | Other |
| Arab/Arab British – Bahrain | |
| Arab/Arab British – Qatar | |
| Arab/Arab British – United Arab Emirates | |
| Arab/Arab British – Oman | |
| Arab/Arab British – Yemen | |
| Arab/Arab British – Saudi Arabia | |

| BMI (kg/m²) |  
| 18-30 | >30.1 |
| <17.9 | |

| Menopausal Stage |  
| Pre-menopausal | Peri- & less than 5 years post-menopause |
| At least 5 yrs post-menopausal | |

| Medication |  
| Contraceptives acceptable | - Vitamin D and Calcium supplementation |
| | - Lipid-lowering drugs |
| | - Digoxin |
| | - Levothyroxine |
| | - Bisphosphonates |
| | - Corticosteroids and Glucocorticoids |
| | - Thiazide diuretics |
| | - Calcium-channel blockers |
| | - Anti-convulsants |
| | - Rifampicin |
| | - Any other medications that may interfere with vitamin D metabolism |

| Medical History |  
| Pregnant or breastfeeding |
| Planning pregnancy in next 6 months |
| Prior/ present history of: |
| - Ischaemic heart disease – i.e. angina, heart attack or stroke |
| - Type 1 and Type 2 Diabetes |
| - Thyroid disease |
| - Osteoporosis, osteopenia or other musculoskeletal disease |
| - Rheumatoid arthritis |
| - Haematological disease |
| - Malignancy |
| - Gastrointestinal disorder, such as Crohns Disease, Coeliac Disease |
| - Liver or kidney disease |
| - Clinical depression or other psychological disorders |
| - Eating disorders |
| - Drug or alcohol abuse within the last 2 years. |

| Social History |  
| Smoking >20/day |
Participants must also give full written informed consent prior to commencing the study.

In addition to the stated exclusion criteria, participants must not:

- Be currently receiving treatment for medical conditions likely to affect vitamin D metabolism
- Regularly use sun-beds
- Have a sun holiday one month prior to commencing the trial or have plans for a sun holiday within the study period.
- Use ‘over-the-counter’ vitamin supplements containing vitamin D
- Be following a weight-reducing diet or under dietary restriction (except vegetarianism)
- Have a known intolerance/allergy to the constituent ingredients of the intervention products

3.3 Withdrawal of participants

All participants will be notified during the consenting process that they are free to withdraw from the trial at any time, without giving a reason.

Participants will be withdrawn from the trial by the Principal Investigator if:

1. Participants experience a reaction to the intervention products (i.e. severe allergic reaction or prolonged gastro-intestinal intolerance)
2. The participant develops a medical condition either prior to entering the study or during, which may adversely affect the outcome of the study.
3. It is clearly demonstrated that the participant is non-compliant to either consuming the intervention product or completing study activities requested of them.

All data prior to subject withdrawal will be used in analysis; unless the participant specifically requests that their data is not to be used. Withdrawn participants will not be replaced as an anticipated drop-out rate of 10% has been accounted for in the recruitment targets.

3.4 Expected duration of trial

The expected duration of the trial is 18 months which includes two 6-month intervention periods. Due to the large number of participants required for the trial, the cohort will be split over the two intervention periods, each participant attending during one intervention period.

Intervention periods will run from November 2011-April 2012 and then November 2012-April 2013.

Each participant will attend three appointments over the course of the trial at baseline (week0), week 6 and week 12.

Final dissemination and publication of the trial results is expected March 2014.
4. Trial procedures

All participant visits will take place at the Clinical Investigation Unit (CIU) based within the Faculty of Health & Medical Sciences, University of Surrey. Trial visits will last approximately 45-60 minutes each (except for Visit 4, which will be a 10 minute appointment only) and all take place in the morning (7am-11am). For the purposes of the blood samples required as part of the trial, participants will be requested to attend fasted (overnight fast). Participants will be offered refreshments at the end of their appointment. Participants will consume two intervention products per day (one biscuit ≈17g and one juice drink ≈220ml) of which one will be a placebo and the other will contain either 600IU/15µg of Vitamin D₂ or Vitamin D₃, or could also be a placebo. Participants will not consume more than 15µg of vitamin D per day (if randomised to receive vitamin D). All intervention products will be manufactured and supplied by Campden BRI. Trial investigators will make regular contact with participants to ensure adequate provision of the intervention products between trial visits; additional products required between visits will be delivered to participants (free-of-charge) by the study team or via courier.

Screening

After receiving the Participant Information Sheet, prospective participants will be contacted via telephone to discuss any questions they may have regarding the study, if they wish to be screened for participation in the study they will then be checked against the study inclusion and exclusion criteria (via the Health & Lifestyle Questionnaire). If the participant is suitable for the study, informed consent will be discussed and they will be invited to the baseline visit.

4.1 Trial visit activities

Visit 1 – Baseline visit (within two weeks of screening interview)

- Participants complete informed consent.
- 4-day diet diary received from participant (diary will be sent to participant prior to appointment, diary will be checked for consistency at visit.)
- Anthropometrics and blood pressure measured, and blood sample taken (serum 25OHD levels, 1,25-dihydroxy vitamin D, lipid profile, glucose, insulin, serum calcium, albumin, parathyroid hormone, C-terminal telopeptide (CTX), full blood count, kidney, thyroid and liver function ≈25ml) with an additional 10ml for genetic profiling and 20 ml for DNA repair analysis, subject to separate consent.
- Peripheral Quantitative Computed Tomography (pQCT) scan of bone mineral composition of forearm (radius).
- Provision of randomly assigned intervention products and dosimeter (to be returned via SAE provided). Follow-up appointment details arranged (including an interim visit telephone appointment).

Visit 2 – Week 6 visit

- Adverse event/compliance interview completed with investigator.
• Anthropometrics and blood pressure measured, and blood sample taken (serum 25OHD levels, 1,25-dihydroxy vitamin D, lipid profile, glucose, insulin, serum calcium, albumin, parathyroid hormone, C-terminal telopeptide (CTX), ≈20ml) with an additional 10ml for genetic profiling and 20 ml for DNA repair analysis, subject to separate consent.

• Provision of assigned intervention products and follow-up appointment details (including an interim visit telephone appointment).

Visit 3 – Week 12 visit

• Adverse event/compliance interview (including diet diary check) completed with investigator.

• Dosimeter and 4-day diet diary received from participant (sent to participant prior to visit).

• Anthropometrics and blood pressure measured, and blood sample taken (serum 25OHD levels, 1,25-dihydroxy vitamin D, lipid profile, glucose, insulin, serum calcium, albumin, parathyroid hormone, C-terminal telopeptide (CTX), ≈20ml) with an additional 10ml and 20 ml for DNA repair analysis for genetic profiling, subject to separate consent.

Visit 4 – Week 16 visit

• 5ml fasted blood sample (serum 25OHD levels, 1,25-dihydroxy vitamin D, vitamin D metabolites)

4.2 Benefits and risks of participation in the D2-D3 Study

To the participant, the main benefit of taking part in the D2-D3 Study is the consistent supplementation of Vitamin D through the winter months where Vitamin D availability is dramatically reduced due to the lack of UV exposure.

Due to the trial being food based, the risk of side-effects is minimal. However, gastrointestinal discomfort or disturbance may occur as the participant adjusts to the consumption of their assigned intervention product. This will be assessed at Visits 2 & 3 via an interview with the trial investigator.

A blood sample must be taken at each trial visit, and due to the nature of the procedure, some light bruising may occur. Occasionally fainting in some individuals can occur relating to venepuncture. To help reduce the risk of this, participants will have their blood sample taken either whilst they are supine on a bed or reclined on a chair that has the capacity to be adapted quickly to allow the participant to lie supine safely if they do become unwell.

The pQCT involves extremely low levels of ionising radiation of which the participants will be exposed to once during the course of the study. The amount of radiation absorbed per scan is 0.22µSv, thus with two scans completed in one session, this equals a total dose of 0.5µSv which is equivalent in radiation exposure to two hours of natural background radiation.

4.3 Blood collection
A trained phlebotomist will take the blood samples required as part of the trial protocol. Medical cover will be available at all times. Approximately 70ml of blood will be taken in total throughout the study plus an additional 90 ml for those choosing to participate in the substudy.

4.4 Genetic profiling

The research activities of the D2-D3 Study have been split into a Main Study and a Sub-Study. The Main Study is the food fortification intervention study where participants are requested to consume the intervention products (containing either vitamin D2 or D3, or placebo) every day for 12 weeks, with the assessment of serum 25OHD as the main study outcome. However, in order to ascertain the mechanism behind vitamin D metabolism and thus support any future results from the D2-D3 Main Study, it is important to analyse genetic polymorphisms that may occur within the study population which may explain any anomalies in the response to vitamin D fortification of their diet (see Section 2.2 Secondary Trial Objectives (iii)-(vi)). Therefore the genetic analysis has been divided into a Sub-Study with a separate consent process due to the sensitivity of the data that will be produced and the need to ensure that participants are fully informed and aware of how their blood samples and subsequent data will be analysed.

4.5 Expenses

Participants will not be remunerated for their participation in the study. However, any travel costs incurred directly relating to the study will be reimbursed, on provision of a valid receipt or evidence of mileage.

5. Ethics and regulatory approval

The trial will be conducted in compliance with the principles of the Declaration of Helsinki (2008), the principles of GCP and in accordance with The Medicines for Human Use (Clinical Trials) Regulations 2004 and Amended Regulations 2006.

This protocol and supporting documents will be submitted for review by the South Coast Research Ethics Committee and the University of Surrey Ethics Committee. Annual progress reports and a final report will be submitted to the ethics committees as defined in their respective regulations.

6. Data Handling

The Principal Investigator will act as custodian for the trial data. The following guidelines will be strictly adhered to:

- Participants data will be anonymised
- All anonymised data will be stored on a password protected computer and if possible, encrypted
- All trial data will be stored and archived as indicated by The Medicines for Human Use (Clinical Trials) Amended Regulations 2006.
7. Publication policy

The results of the study will be reported and disseminated to the scientific community via peer-reviewed journals and international conferences. The general public will also be engaged via the release of results to the local and national media, relevant charities and community networks.

8. Finance

Full funding to conduct the D2-D3 Study is provided by the Biotechnology and Biological Sciences Research Council’s Diet and Health Research Industry Club (BBSRC DRINC).

9. Signatures

________________________________ _____________________
Principal Investigator  Date
D2-D3 Study Consent Form (Main Study)

Participant Number:

• I (name) ___________________________ voluntarily agree to take part in the above named research study conducted by the University of Surrey.

• I have read and understood the Main Study Participant Information Sheet provided (Date 19/10/2011; Version 1.6). I have been given a full explanation by the investigator of the nature, purpose, location and likely duration of the study, and of what I will be expected to do. I have been advised about any discomfort and possible ill-effects on my health and well-being which may result. I have been given the opportunity to ask questions on all aspects of the study and have understood the advice and information given as a result.

• I agree to comply with any instruction given to me during the study and to co-operate fully with the investigators. I shall inform them immediately if I suffer any deterioration of any kind in my health or well-being, or experience any unexpected or unusual symptoms.

• I agree to the investigators contacting my general practitioner about my participation in the study, and I authorise my GP to disclose details of my relevant medical or drug history (if necessary), in confidence.

• I consent to my personal data, as outlined in the accompanying information sheet, being used for this study and other research. I agree that anonymised data collected may be shared with other researchers or interested parties. I understand that all personal data relating to volunteers is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998).

• I understand that in the event of my suffering a significant and enduring injury (including illness or disease) as a direct result of my participation in the study, compensation will be paid to me by the University of Surrey subject to certain provisos and limitations. The amount of compensation will be appropriate to the nature, severity and persistence of the injury, and will, in general terms, be consistent with the amount of damages commonly awarded for similar injury by an English court in cases where the liability has been admitted.

• I understand that I am free to withdraw from the study at any time without needing to justify my decision and without prejudice.

• I confirm that I have read and understood the above and freely consent to participating in this study. I have been given adequate time to consider my participation and agree to comply with the instructions and restrictions of the study.

.................................................................  …………………..  ………………………….
Name of participant (BLOCK CAPITALS)  Date  Signature

.................................................................  …………………..  ………………………….
Name of witness (if appropriate)  Date  Signature

.................................................................  …………………..  ………………………….
Name of Researcher/person taking consent  Date  Signature

Copies: 1 for participant; 1 (original) for investigator site file
D2-D3 Study Consent Form (Main Study) Version 1.6 (19/10/2011) NHS REC Ref: 11/LO/0708
INTRODUCTION

We would like you to keep this diary of everything you eat and drink over the next seven days. This is a very important part of the study and will add greatly to the information you have already given us about your usual diet.

Completing this diary carefully will take you some time, but the valuable record given by you, and many others in this study will help us understand fully the effects of diet on health.

It is very important that you do not adjust what you eat and drink just because you are keeping a record. Please continue to eat whatever you would eat normally.

Please provide us with as much detail as you possibly can.

If you have any queries please ring:

01483 689222

or

d2d3@surrey.ac.uk

School of Biomedical & Molecular Sciences
University of Surrey, Guildford, Surrey GU2 7XH
e-mail: s.lanham-new@surrey.ac.uk

When you have completed the food diary, please post the booklet back to us in the envelope provided.

Thank you

GENERAL INSTRUCTIONS

1. As you will see, each day is marked in sections, beginning with first thing in the morning and ending with bedtime. For each part of the day:
   - Write down all food and drink consumed, the amounts and a description.
   - If nothing is eaten or drunk, draw a line through that section.
   - At the end of each day there is a list of snacks and drinks that can easily be forgotten. Please write any extra items in here if you have not already recorded them in some other part of the day.
   - If you prepare a recipe, please write it in the recipe box provided at the end of each day.

2. Please try to record everything at the time of eating, not from memory at the end of the day.

3. Please read pages 3-8 for help in describing the foods and drinks you have eaten. Pages 9-15 include a range of photographs and page 16 shows an example of part of a completed diary.

4. Give brand and full name of products from packaging. Many commercial foods have weights printed on them, so please use these to show how much you ate.

5. Please answer the questions at the back of the diary (pages 45-48), after you have completed the seven days.
**DETAILED INSTRUCTIONS**

The following section is a list of popular foods and drinks. Next to each item is the sort of thing we need to know so that we can tell what it is made of and how much you had. This list cannot cover all foods and drinks, so if anything that you have eaten is missing try to relate it to a similar item. Please give as much detail as you can. For an example of how you might describe foods you have eaten see page 16.

Please try to state what sort of oil or fat was used for baking, frying etc.

State clearly whether spread was used on crackers and biscuits as well as on bread, rolls, toast and in sandwiches.

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description &amp; Preparation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Homemade dishes</strong></td>
<td>Describe as fully as possible, include name of dish; give recipe or ingredients, including amounts if known</td>
<td>Tablespoons; one of the suitable photos</td>
</tr>
<tr>
<td><strong>Ready-made meals</strong></td>
<td>Give name of dish as described on pack with brand; describe main ingredients and enclose label e.g. beef lasagne, deep pan pizza, fish pie etc.</td>
<td>Weight from packet including proportion of pack eaten (all or half?) Tablespoons; one of the suitable photos</td>
</tr>
<tr>
<td><strong>Meals eaten away from home or take-away meals</strong></td>
<td>Please describe all dishes and give main ingredients e.g. lamb tikka masala and pilau rice, other indian and oriental dishes, fish and chips, burgers, pizza etc.</td>
<td>Proportion of takeaway or restaurant carton Describe meal size and dimensions where appropriate Tablespoons; one of the suitable photos</td>
</tr>
<tr>
<td><strong>BEVERAGES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alcoholic drinks e.g. beer, lager, cider, sherry, wine, spirits and liqueurs</td>
<td>Describe type and give alcohol content especially for beers, lagers and wines</td>
<td>Number of pints Number and size of cans, bottles or glasses Number of measures Volume (fl. oz. or ml.)</td>
</tr>
<tr>
<td>Fruit juice</td>
<td>Without added sugar</td>
<td>Glasses, cartons, cans or bottles with volume</td>
</tr>
<tr>
<td>Fruit drinks</td>
<td>With added sugar</td>
<td></td>
</tr>
<tr>
<td>Soft drinks</td>
<td>Brand name, regular or diet or low calorie</td>
<td></td>
</tr>
<tr>
<td>Coffee</td>
<td>Instant or ground; decaffeinated or decaffeinated; with milk or sugar</td>
<td>Cups or mugs Volume if available</td>
</tr>
</tbody>
</table>

| **Tea**                             | Tea leaves or tea bag, with milk or sugar. If instant: black or white, sweetened or not                    | Cups or mugs Volume if available                                                                           |
| **Milk based or hot chocolate type drinks** | Name or type of drink; regular, reduced fat or low sugar Type of milk used                               | Cups or mugs Volume if available                                                                           |
| **Water**                           | Tap, bottled or filtered                                                                                  | Glass, tumbler; volume                                                                                    |

| **BISCUITS / CRACKERS**              |                                                                                                             |                                                        |
| **Sweet biscuits**                  | Brand and full product name plus description e.g. sandwich, wafer, chocolate half-coated, full-coated, cream-filled Ingredients if homemade                                                                               | Number of biscuits and size                                                                              |
| **Crackers, crisp bread, savoury biscuits** | Brand and full product name plus description e.g. Carr's water biscuits, Original Ryvita, Jacob's Choicegrain | Number of crackers and size                                                                              |

| **BREAD**                           |                                                                                                             |                                                        |
| **Bread**                           | White, brown, granary, wholemeal, containing seeds, ciabatta, focaccia, french type, baguette. Was the loaf pre-sliced or hand-cut?                                                                          | Size of loaf: large or small Thickness of slice Number of slices                                           |
| **Rolls or buns**                   | Describe rolls: crusty, soft, baps, petit pain, finger                                                  | Size of rolls and number                                                                                 |
| **Sandwiches**                      | Remember to describe type and amount of spread and filling                                               | Number of rolls or slices of bread                                                                       |

<p>| <strong>BREAKFAST CEREALS</strong>               |                                                                                                             |                                                        |
| <strong>Breakfast cereal</strong>                | Brand and full name e.g. Jordan's Natural Muesli, Sainsbury's Malties Remember to describe milk and sugar added separately | Photo 1 Tablespoons Milk on cereal: large, medium or small amount                                         |
| <strong>Porridge or Ready Brek</strong>          | Porridge oats or Ready Brek Type of milk used to make it or was water used? Remember to describe milk and sugar added separately                         | Photo 1 Amounts of ingredients                                                                           |
| <strong>Bran:</strong> wheat bran, wheat germ and bran | Added separately to breakfast cereal or mixed with other foods such as porridge. Please describe type and brand                           | Dessertspoons or tablespoons                                                                            |</p>
<table>
<thead>
<tr>
<th>BUTTER, MARGARINES, FATS &amp; OILS</th>
<th><strong>FISH</strong></th>
<th><strong>FRUIT</strong></th>
<th><strong>MEAT</strong></th>
<th><strong>MILK/DAIRY</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Butter, spreads or margarines</strong></td>
<td>Please give specific brand, full name as described on packaging plus the percentage (%) fat if known.</td>
<td><strong>Type</strong></td>
<td><strong>Type</strong></td>
<td><strong>Whole, semi-skimmed or skimmed; percentage (%) fat if known Pasteurised, UHT or sterilised</strong></td>
</tr>
<tr>
<td></td>
<td>Photo 18 for spread on bread or rolls For crackers and biscuits describe thickness of spread.</td>
<td><strong>Fresh, frozen or canned, cooking method; from fish and chip shop, homemade or commercial; battered or breadcrumb</strong></td>
<td><strong>Type; fresh (was skin eaten or not?), frozen, dried, stewed with or without sugar Canned in syrup or juice</strong></td>
<td><strong>Volume in fl.oz. or ml.</strong></td>
</tr>
<tr>
<td><strong>Oils</strong></td>
<td><strong>Weight (with or without bones/skin?); size of whole or piece of fish Photo 6</strong></td>
<td><strong>Number of whole fruits</strong></td>
<td><strong>Weight (with or without skin)</strong></td>
<td><strong>Teaspoons; volume of made up milk</strong></td>
</tr>
<tr>
<td><strong>CAKES</strong></td>
<td><strong>Tablespoons</strong></td>
<td><strong>Weight of</strong></td>
<td><strong>Weight (raw or cooked)</strong></td>
<td><strong>Teaspoons; volume of</strong></td>
</tr>
<tr>
<td><strong>Cakes, scones and sweet buns, pies and pastries</strong></td>
<td><strong>Homemade – describe ingredients and recipe</strong> Commercial – give brand and product name with description Does cake contain filling e.g. whipped cream, butter icing or have a coating or covering? Are pies made with pastry top and bottom?</td>
<td><strong>Weight; number and size or thickness of slices Photo 4</strong></td>
<td><strong>Number and size</strong></td>
<td><strong>Teaspoons; Individual cartons or sachets</strong></td>
</tr>
<tr>
<td></td>
<td>Proportion of whole cake or pie Size of slice or individual cake Photos 15 and 16 for cake Photo 3 for pies or flans</td>
<td><strong>Back, middle, streaky; unsmoked or smoked Rashers or chops</strong></td>
<td><strong>Number and size</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td><strong>CHEESE</strong></td>
<td><strong>Are scones or cakes spread with butter, margarine and/or jam?</strong></td>
<td><strong>Weight (raw or cooked)</strong></td>
<td><strong>Weight (raw or cooked)</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td><strong>Hard cheese (includes Brie, Danish Blue etc.)</strong></td>
<td><strong>Specify type e.g. Cheddar, Wensleydale, Brie</strong></td>
<td><strong>Number and size</strong></td>
<td><strong>Weight (raw or cooked)</strong></td>
<td><strong>Tablespoons; pack weight</strong></td>
</tr>
<tr>
<td></td>
<td>Photo 2 (amount eaten is equal to the slice OR the chunk OR the grated cheese) Number and size of slices or chunks</td>
<td><strong>Type, cooking method</strong></td>
<td><strong>Weight (raw or cooked)</strong></td>
<td><strong>Tablespoons; pack weight</strong></td>
</tr>
<tr>
<td><strong>Philadelphia type soft cheese or cheese spread</strong></td>
<td><strong>Regular or reduced fat cheese</strong> Specify brand and fat content</td>
<td><strong>Recipe or brand and product name with ingredients Photo 5, 19, or 20</strong></td>
<td><strong>Number and size</strong></td>
<td><strong>Tablespoons; pack weight</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Thick or thin spread Teaspoons</strong></td>
<td><strong>Photo 5, 19, or 20</strong></td>
<td><strong>Tablespoons; pack weight</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td><strong>DESSERT/PUDDINGS</strong></td>
<td><strong>Puddings</strong></td>
<td><strong>Coffee or tea creamer or whiter</strong></td>
<td><strong>Brand and product name e.g. Coffee mate Please state if powder or liquid</strong></td>
<td><strong>Teaspoons; Individual cartons or sachets</strong></td>
</tr>
<tr>
<td></td>
<td>Describe type and ingredients e.g. apple crumble, raspberry cheesecake with biscuit base, dairy cream trifle with banana Served with custard, ice cream, cream or yogurt? (see milk)</td>
<td><strong>Brand and product name or description, fat content as on carton</strong></td>
<td><strong>Tablespoons; size of carton (g, or ml.)</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td><strong>EGGS</strong></td>
<td><strong>Eggs and egg dishes</strong></td>
<td><strong>Liquid, whipped or aerosol</strong></td>
<td><strong>Tablespoons</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td></td>
<td>Boiled, poached, fried, scrambled, omelette plus topping or other ingredients Was fat or oil used in cooking? Give type of fat or oil used</td>
<td><strong>Brand and product name; regular, reduced fat or made with cream</strong></td>
<td><strong>Scoops</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Size of eggs Number of eggs consumed</strong></td>
<td><strong>Tablespoons</strong></td>
<td><strong>Scoops</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td></td>
<td><strong>Photo 17; tablespoons Size of slice; weight of carton for commercial items</strong></td>
<td><strong>Tablespoons</strong></td>
<td><strong>Scoops</strong></td>
<td><strong>Tablespoons</strong></td>
</tr>
<tr>
<td><strong>PASTA</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta and spaghetti incl. filled pasta</td>
<td>Dried or fresh pasta; white or wholemeal; describe type e.g. fusilli or tagliatelle Filled pasta e.g. Tortelloni with spinach and ricotta</td>
<td>Weight (raw or cooked) Photo 9 Proportion of packet weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta dishes</td>
<td>Lasagne, cannelloni or pasta bakes; give recipe and ingredients for homemade; brand, product name and description for commercial</td>
<td>Photo 20 Packet weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta sauce</td>
<td>Describe sauce type and ingredients</td>
<td>Tablespoons Volume or weight of commercial product</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>RICE</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice</td>
<td>White or brown, long grain or basmati</td>
<td>Photo 8 Weight (raw or cooked)</td>
</tr>
<tr>
<td>Rice dishes</td>
<td>Give recipe and ingredients for homemade; brand, product name and description for commercial</td>
<td>Photo 8: tablespoons Packet weight and proportion eaten</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SAUCES &amp; SOUPS</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sauces and ketchups including dips</td>
<td>Describe brand and product name or recipe and ingredients</td>
<td>Tablespoon or teaspoons Volume or weight of commercial product</td>
</tr>
<tr>
<td>Soups</td>
<td>Describe type and ingredients Is soup homemade, canned, condensed, dried packet, instant, fresh/carton or low calorie?</td>
<td>Bowls, cups or mugs Volume in fl.oz. or ml. Weight of can and proportion eaten</td>
</tr>
<tr>
<td>Gravy</td>
<td>Describe brand and product name or recipe and ingredients Made with cornflour, bisto powder, granules; with or without added meat juices, stock or vegetable juice</td>
<td>Tablespoons Volume in ml. or fl.oz.</td>
</tr>
<tr>
<td>Dressings</td>
<td>Type and ingredients; brand and product name; regular, reduced fat or fat free</td>
<td>Tablespoons or teaspoons</td>
</tr>
<tr>
<td>Mayonnaise</td>
<td>Regular or reduced fat</td>
<td>Tablespoons etc.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SAVOURY DISHES</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pies, flans and quiches Pizza Pancakes Sausage rolls Filled tortillas or burritos</td>
<td>Describe dish and ingredients, brand and product name</td>
<td>Product weight and proportion eaten Number of slices or individual items eaten</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SAVOURY SNACKS</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Crisps and snacks Nuts</td>
<td>Brand name and description Type; fresh or roasted; salted or unsalted</td>
<td>Weight of packet Number of items eaten</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SPREADS &amp; CONDIMENTS</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Jams, other preserves and spreads</td>
<td>Brand name and type of spread Jam, honey, marmalade Peanut butter, other nut butters Chocolate spread Marmite and savoury spread</td>
<td>Thin, medium or thick spread</td>
</tr>
<tr>
<td>Salt, pepper, mustard</td>
<td>Describe type</td>
<td>Sprinkle; teaspoons</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>SUGARS &amp; CONFECTIONERY</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Sweets and chocolate</td>
<td>Describe type and brand</td>
<td>Weight; number of pieces, whole bars or individual sweets</td>
</tr>
<tr>
<td>Sugars and sweeteners</td>
<td>Type of sugar Brand and type of sweetener</td>
<td>Teaspoons Tablets or spoons</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>VEGETABLES (including herbs)</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables and salad including lentils, beans and baked beans</td>
<td>Type of vegetables; fresh, frozen or canned; cooking method or raw If roasted was fat added? Was butter, sauce or dressing added?</td>
<td>Photo 12,13 or 14 Number of whole vegetables Tablespoons</td>
</tr>
<tr>
<td>Vegetable dishes including dishes with potato, beans, lentils or pulses</td>
<td>Recipe or brand and product name with ingredients</td>
<td>Photo 5 or 20 Weight of commercial dish</td>
</tr>
<tr>
<td>Potatoes</td>
<td>Boiled; roasted with or without fat; fried; sautéed; mashed with or without added fat or milk</td>
<td>Photo 10 or 11</td>
</tr>
<tr>
<td>Chips</td>
<td>Homemade; commercial e.g. oven chips; takeaway Size and cut of chip</td>
<td>Photo 7</td>
</tr>
<tr>
<td>Herbs and spices</td>
<td>Fresh or dried</td>
<td>Teaspoons or other spoons; leaves; sprigs</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>VEGETARIAN</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetarian products and dishes with Quorn, soya or TVP or tofu</td>
<td>Describe dish or product and ingredients, brand and product name e.g. Quorn sausages, Vegetable stir-fry with tofu</td>
<td>Weight from packaging Number of items Number of slices of meat substitute Photo 5 Tablespoons</td>
</tr>
</tbody>
</table>
Please choose an appropriate photo to indicate the portion size you have eaten. To help you make this choice, there are some notes below the photos. Write down the picture number and size nearest to your own helping e.g. 2a, 3b or 1c.

The large white circle in the background shows the actual size of the 10” dinner plates used in the photos. Items such as the cake are photographed on a 7” tea plate.

Refer to the detailed instructions on pages 3 - 8 where * is indicated.

<table>
<thead>
<tr>
<th>1a</th>
<th>1b</th>
<th>1c</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Cereal" /></td>
<td><img src="image2.png" alt="Cereal" /></td>
<td><img src="image3.png" alt="Cereal" /></td>
</tr>
<tr>
<td>Suitable for – Corn flakes and other breakfast cereals</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2a</th>
<th>2b</th>
<th>2c</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image4.png" alt="Cheese" /></td>
<td><img src="image5.png" alt="Cheese" /></td>
<td><img src="image6.png" alt="Cheese" /></td>
</tr>
</tbody>
</table>
| Suitable for – Cheese  
Not for – Butter, margarines and spreads (see photo 18)  
**PLEASE NOTE:** When choosing one of the photos above, the amount you eat is equal to either the slice OR the chunk OR the grated cheese on one plate |

<table>
<thead>
<tr>
<th>3a</th>
<th>3b</th>
<th>3c</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image7.png" alt="Pizza" /></td>
<td><img src="image8.png" alt="Pizza" /></td>
<td><img src="image9.png" alt="Pizza" /></td>
</tr>
</tbody>
</table>
| Suitable for – Quiches, flans, savoury or sweet pies and pizza  
Not for – Cakes (see photos 15 and 16) |

<table>
<thead>
<tr>
<th>4a</th>
<th>4b</th>
<th>4c</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image10.png" alt="Meat" /></td>
<td><img src="image11.png" alt="Meat" /></td>
<td><img src="image12.png" alt="Meat" /></td>
</tr>
</tbody>
</table>
| Suitable for – Hot or cold sliced meats, e.g. roast meat, ham or gammon  
Not for – Chops, steaks or bacon rashers * |

<table>
<thead>
<tr>
<th>5a</th>
<th>5b</th>
<th>5c</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image13.png" alt="Stew" /></td>
<td><img src="image14.png" alt="Stew" /></td>
<td><img src="image15.png" alt="Stew" /></td>
</tr>
</tbody>
</table>
| Suitable for – Vegetable stews or meat stews and casseroles WITH vegetables, also bolognaise sauce  
Not for – Meat stews WITHOUT vegetables (see photo 19) |
Appendix 29

Suitable for - Boiled or roast potato

Suitable for - Mashed potato

Suitable for - Boiled pasta and noodles plus pastas and noodle dishes

Suitable for - Boiled rice and rice dishes

Suitable for - Fish including fish in breadcrumbs or batter

Not for - Chops or steaks *
<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>15c</td>
<td>Sponge cake and other similar cakes</td>
</tr>
<tr>
<td>15b</td>
<td>Quiches, flans and savoury pies (see photo 3)</td>
</tr>
<tr>
<td>16a</td>
<td>Suitable for - Fruit cake and other cake types with same shape</td>
</tr>
<tr>
<td>16b</td>
<td>Not for - Meat (see photo 4)</td>
</tr>
<tr>
<td>17a</td>
<td>Suitable for - Fruit crumble and other puddings and desserts</td>
</tr>
<tr>
<td>17b</td>
<td>Not for - Puddings WITH custard, sauce, yoghurt or ice cream combined</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Plate</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>12c</td>
<td>Baked beans and peas</td>
</tr>
<tr>
<td>12b</td>
<td>Suitable for - Carrots and other similar vegetables</td>
</tr>
<tr>
<td>12a</td>
<td>Suitable for - Cabbage, other leafy vegetables and salads</td>
</tr>
<tr>
<td>13c</td>
<td>Not for - Beans (see photo 12)</td>
</tr>
<tr>
<td>13b</td>
<td>Suitable for - Carrots and other similar vegetables</td>
</tr>
<tr>
<td>13a</td>
<td>Suitable for - Peas (see photo 12)</td>
</tr>
<tr>
<td>14c</td>
<td>Suitable for - Cabbage, other leafy vegetables and salads</td>
</tr>
<tr>
<td>14b</td>
<td>Not for - Peas (see photo 12)</td>
</tr>
<tr>
<td>14a</td>
<td>Suitable for - Carrots and other similar vegetables</td>
</tr>
</tbody>
</table>
### Food/Drink Description and Preparation

#### LUNCH

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BEEF CASSEROLE</strong></td>
<td>Beef casserole (onion and carrots)</td>
<td>Photo 5b</td>
</tr>
<tr>
<td><strong>POTATOES</strong></td>
<td>Mashed potatoes</td>
<td>2 scoops</td>
</tr>
<tr>
<td><strong>VEGETABLES</strong></td>
<td>Boiled cabbage</td>
<td>Photo 14a</td>
</tr>
<tr>
<td><strong>DESSERT</strong></td>
<td>Rhubarb crumble</td>
<td>Photo 17b</td>
</tr>
<tr>
<td><strong>TEA</strong></td>
<td>Tea bag</td>
<td>2 small ladles</td>
</tr>
<tr>
<td><strong>MILK</strong></td>
<td>Milk - semi-skimmed (no sugar)</td>
<td>1 plastic cup</td>
</tr>
<tr>
<td><strong>Amount</strong></td>
<td></td>
<td>1 tbsp</td>
</tr>
</tbody>
</table>

#### TEA - BETWEEN LUNCH TIME AND EVENING MEAL

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SANDWICH</strong></td>
<td>Brown bread, large sliced loaf</td>
<td>1 medium slice</td>
</tr>
<tr>
<td><strong>SPREAD</strong></td>
<td>St. Ivel Utterly Butterly</td>
<td>thick spread</td>
</tr>
<tr>
<td><strong>FILLING</strong></td>
<td>Grated cheddar cheese and tomato</td>
<td>1/2 x Photo 2c</td>
</tr>
<tr>
<td><strong>APPLE</strong></td>
<td>Small Braeburn - ate skin</td>
<td>2 slices</td>
</tr>
<tr>
<td><strong>TEA</strong></td>
<td>As lunch with whole milk</td>
<td>1 fruit</td>
</tr>
<tr>
<td><strong>CHOCOLATE</strong></td>
<td>Cadbury's Dairy Milk - small bar</td>
<td>1 large mug</td>
</tr>
<tr>
<td><strong>Amount</strong></td>
<td></td>
<td>3 tbsp milk</td>
</tr>
<tr>
<td><strong>Amount</strong></td>
<td></td>
<td>1 (49g)</td>
</tr>
</tbody>
</table>

#### EVENING MEAL

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CHICKEN &amp; VEGETABLE STIR-FRY</strong></td>
<td>Skinless and boneless chicken breast, packaged, 300 gram raw Vegetable oil 1 large carrot, 2 spring onions 1 small courgette, 1 med. red pepper, 4 oz button mushrooms 2 tsp grated ginger, 1 tbsp soy sauce, 1 tbsp sherry White rice, boiled Muller Fruit Corner - strawberry Cabernet Sauvignon (14.5% alcohol)</td>
<td>4 heaped tbsp 1 carton (175g) 1 large wine glass (270 ml)</td>
</tr>
<tr>
<td>DATE / / DAY OF THE WEEK:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BEFORE BREAKFAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food/Drink</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BREAKFAST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food/Drink</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MID MORNING – between breakfast time &amp; lunch time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food/Drink</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LUNCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food/Drink</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>TEA – between lunch time &amp; the evening meal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food/Drink</td>
</tr>
</tbody>
</table>

Appendix 30
### EVENING MEAL

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
</table>

### LATER EVENING – up to last thing at night

<table>
<thead>
<tr>
<th>Food/Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
</table>

### ANYTHING ELSE?
Between meal snacks and drinks NOT already written in before

<table>
<thead>
<tr>
<th>Food / Drink</th>
<th>Description and Preparation</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chocolate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toffees, sweets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crisps, peanuts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other snacks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beer, wine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sherry, spirits</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other cold drinks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tea, coffee</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other hot drinks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ice cream</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anything else?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Space to write in the recipe or ingredients of any home-made dishes, take-away meals etc. that you have mentioned but not described previously. Where applicable, please list amounts of ingredients and brand names. Please indicate the amount or proportion actually consumed by yourself.
GENERAL QUESTIONS ABOUT YOUR FOOD/DRINK LAST WEEK

1. Which type of milk did you most often use last week? **Select one only.**
   - Whole/full cream
   - Semi-skimmed
   - Skimmed/fat free

   Do you know the fat percentage (%) of your milk?:
   - Yes

   Was this milk: pasteurized? UHT? sterilized? dried?

2. How much milk did you usually have in tea, coffee and on your cereal?
   - Tea: A lot
   - Coffee: A lot
   - Cereal: A lot

3. Did you drink decaffeinated tea or coffee?
   - Tea: Always
   - Coffee: Always

4. Which types of fat did you use last week for baking, frying, spreading and on salads? **If you are not sure which category to indicate, check packaging for the exact name, fat content and brand and fill in this information.**

<table>
<thead>
<tr>
<th>Type of fat, spread or margarine</th>
<th>Brand and name of product</th>
<th>Spreading</th>
<th>Frying</th>
<th>Baking</th>
<th>Salads</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spreadable butter</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dairy spread (e.g. I can't believe it's not butter)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polyunsaturated spread (sunflower, soya or vegan)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

5. Which type of bread did you eat most often last week? **Select one only.**
   - White
   - Granary
   - Wholemeal
   - Other:

<table>
<thead>
<tr>
<th>Soft grain</th>
<th>Brown</th>
<th>Wheatgerm</th>
</tr>
</thead>
</table>

(continued....)
6. Did you eat butter, margarine or spread last week? Please tick boxes below to show whether you ate it on toast, bread, sandwiches, in rolls or on crackers:

<table>
<thead>
<tr>
<th></th>
<th>Toast</th>
<th>Bread</th>
<th>Sandwiches</th>
<th>Rolls</th>
<th>Crackers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Always</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sometimes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Don't know</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

7. How thickly did you spread butter, margarine etc. on bread or crackers?

- Thick
- Medium
- Thin
- None

8. If you ate grilled, fried, barbecued or roast meat last week, how well cooked was it? Please tick the boxes.

<table>
<thead>
<tr>
<th></th>
<th>Beef, lamb, pork</th>
<th>Poultry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well done or dark brown</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lightly cooked or rare</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did not eat meats cooked by these methods</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did not eat these meats</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

9. If you ate meat last week, what did you do with the visible fat? Please note that meat includes beef, lamb, pork, ham and bacon.

- Ate all of the fat
- Ate some of the fat
- Did not eat meat
- Ate most of the fat
- Ate as little as possible
- No fat eaten

10. If you ate poultry last week, did you eat the skin? Please note that poultry includes chicken, duck, goose and game birds.

- Yes
- Sometimes
- No
- Did not eat poultry

11. If you had gravy last week, were the meat juices, pan residues or dripping put into the gravy?

- Yes
- No
- Sometimes
- Don’t know
- Did not eat gravy

12. Was salt usually added to your food during cooking last week?

- Yes
- No
- Don’t know
- Did you usually add salt to your food at the table last week?

- Yes
- No
- Don’t know
- Did you regularly use a salt substitute (e.g. LoSalt) last week?

- Yes
- No
- Don’t know
- If YES, which brand?

13. Did you eat the skin on fruit? Please tick boxes.

<table>
<thead>
<tr>
<th></th>
<th>Apple</th>
<th>Pear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin eaten</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin not eaten</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fruit not eaten</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
14. Please name any vitamins, minerals or other food supplements taken on each day of last week. Please write down all the details from each packet/container or enclose label(s). Give the number of tablets taken on each day.

<table>
<thead>
<tr>
<th>Brand</th>
<th>Name</th>
<th>Strength</th>
<th>Tablet capsules</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 5</th>
<th>Day 6</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boots</td>
<td>High strength vitamin C</td>
<td>1000 mg</td>
<td>Tablet</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

15. Which types of water did you consume last week? Please give information for both HOT and COLD drinks.

<table>
<thead>
<tr>
<th>Water type</th>
<th>Hot drinks</th>
<th>Cold drinks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water (unfiltered)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filtered water – hard water filter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filtered water – other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottled water – brand:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other water – brand:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

16. Were any of the following foods which you ate last week produced organically (without pesticides)? Please tick the necessary box(es).

- Vegetables, homegrown
- Fruit, homegrown
- Milk and dairy products
- Meat
- Vegetables, purchased
- Fruit, purchased
- Cereal or cereal products, bread
- No organic foods eaten

This space has been left for you to tell us about anything else which you feel is important about your food/drink intake last week.

Please post the diary back to us.

Thank you very much for your help in completing such a detailed record.
Adverse Event and Compliance Interview – Visits 2 & 3

Ergocalciferol (Vitamin D₂) vs. Cholecalciferol (Vitamin D₃) Food Fortification: Comparative Efficiency in raising 25OHD Status in Asian & Caucasian Women and Mechanisms of Action (D2-D3 Study)

Participant Number:

<table>
<thead>
<tr>
<th>Visit 2</th>
<th>Date:</th>
<th>Biscuit</th>
<th>Juice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity of IP given at Visit 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantity of IP returned at Visit 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Packets returned? Count:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantity of IP consumed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quantity of IP not consumed</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If quantity of IP not consumed is greater than 10% of initial allocation, please give reasons.

Reduced consumption linked to Adverse Event? YES/NO
If yes, please complete Adverse Event Log.

Are solutions available to assist with compliance? YES/NO
If yes, please state solution. If no, please state barriers to compliance.

Is the participant happy to continue with the intervention? YES/NO
If YES, allocate intervention product - _____ juice cartons, _____ biscuits
If NO, participant may withdraw from D2-D3 Study if they so wish.

Interview completed by: Date:
<table>
<thead>
<tr>
<th>Visit 3</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Biscuit</td>
</tr>
<tr>
<td>Quantity of IP given at Visit 2</td>
<td></td>
</tr>
<tr>
<td>Quantity of IP returned at Visit 3</td>
<td></td>
</tr>
<tr>
<td>Packets returned? Count:</td>
<td></td>
</tr>
<tr>
<td>Quantity of IP consumed</td>
<td></td>
</tr>
<tr>
<td>Quantity of IP not consumed</td>
<td></td>
</tr>
</tbody>
</table>

If quantity of IP **not consumed** is greater than 10% of allocation from Visit 2, please give reasons.

Reduced consumption linked to Adverse Event? YES/NO
If yes, please complete Adverse Event Log.

Final comments?

Interview completed by: Date:
**D2-D3 Study: Adverse Events Log**

All changes in health over intervention period that may affect compliance to be recorded*

<table>
<thead>
<tr>
<th>No</th>
<th>Event Name</th>
<th>Start date (DD/MMM/YYYY)</th>
<th>Stop date (DD/MMM/YYYY)</th>
<th>Medication required? (Give detail)</th>
<th>Intensity</th>
<th>Intervention stopped?</th>
<th>Outcome</th>
<th>Relationship to Intervention Product</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td>0 - Mild</td>
<td>1 - Temporarily Interrupted</td>
<td>0 - Resolved</td>
<td>0 - Unlikely</td>
</tr>
<tr>
<td>2.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td>1 - Moderate</td>
<td>2 - Permanently withdrawn</td>
<td>1 - Resolved</td>
<td>1 – Possibly</td>
</tr>
<tr>
<td>3.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td>2 - Severe</td>
<td>2 - Not resolved</td>
<td>2 - Not resolved</td>
<td>2 – Likely</td>
</tr>
<tr>
<td>4.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td>3 - Definitely</td>
</tr>
<tr>
<td>5.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.</td>
<td></td>
<td><em><strong>/</strong>__/</em>___</td>
<td><strong><strong>/</strong></strong>/____</td>
<td>No</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For example, conditions may include colds and 'flu, gastrointestinal issues, prolonged headache, food intolerance etc*
Standard procedures to use with Dietplan6

• Setting up which nutrients to analyse:
  - “tasks” → “which nutrients to display” → select ones to display →
    - Follow nutrient list provided, in the exact same order
  - “save as” – assign a unique name such as D2D3 Study

• Starting an analysis:
  - “file” → “new analysis” → complete the Registration No. (ie D2D3_YOURNAME) and Surname (YOUR NAME) →
    click continue
  - Selection tab window –
    - Can record multiple entries for one individual or study.
    - To start new assessment (you may need to scroll down to find ‘new’) →
      Double click “new” → from drop down menu select “intake diary” → give the file a title (e.g. D2D3_1406A_V3) → select number of days for the diary (4 days) → select number of meals for each day (6 meals, 1 course per meal) → click continue
      → “file” → “save as”* →
      “ok” → it returns you to the registration tab just click back through and select the assessment from the list → start to add foods
  - * you MUST click “save as” before proceeding or you will be unable to save the foods after you have entered them
    - To continue/edit a previous assessment → click ‘open analysis’ and search D2D3_YOURNAME, double click on the assessment and then click continue.

• Entering foods:
  - Yellow shaded areas are read only – White areas can be edited
  - Click on “add item” – or Ctrl+F
  - Untick “using whole words only” – makes searching quicker as allows searching using just a few letters rather than the whole word
  - Tick “repeat this dialog until cancelled” allows many foods to be added to a meal until closed using the cross in the top right corner
  - Enter food into “search for” box – either the food, food code or own food code
D2D3 Study Procedure

- Select which food is required – when there is more than one option for the food always go with –
  1st – UKN (UK nutrient databank) (integrates all other sources)
  2nd – IDS (integrated dataset) (2008)

- Select either the portion size or add the weight of food. If the participant hasn’t specified a portion size always use a medium serving size

- Click “add”

- When finished a meal close the dialog box

- Click “save”

- Use “<prev” and “next>” buttons to navigate between days/meals or click on the actual meal in the navigate box

- If the same foods have been eaten → highlight the line in the bottom box → “edit” → “copy” → move to the meal where required → “edit” → “paste” – remember to change the portion size if required

- Where a participant has specified a particular brand of food always find the nutritional information for that particular food and enter it into the analysis and do not use generic alternatives

- Try to be consistent between diaries if using generic foods – e.g. if they have just stated semi-skimmed milk always use the same milk food code across all the diaries (e.g. code 12-312) – useful to keep a record of commonly used food codes

- Adding own foods:
  - “data” → “add own food” →
    - Insert a ref code (Keep this short e.g. initials and a number)
    - Add the correct food name into the “name” section
    - From packets enter nutrients into:
      - Energy (kcal) kilocal (from proximates section)
      - Energy (kJ) kilojoules (from proximates section)
      - Protein (from proximates section)
      - Available Carbohydrate (from proximates section)
      - Total Sugars (from carbohydrate components section)
      - Fat (from proximates section)

D2D3 Study Procedure

- Saturated fatty acids (from fat components section)
- Total Dietary fibre (AOAC) (from proximates section)
- Sodium (from minerals – macroelements section)

  o Click “save”
  o Do not add values into the edible proportion section unless this is correct (e.g. if adding values for a whole fish, bones are not eaten therefore the edible portion is less) this is not where portion consumed is put
  o Always check units on packets match the units in the program and convert if necessary (particularly important for sodium – often in grams on packets and the program requires it in milligrams)
  o Nutrients can be inputted per any amount of grams of food (bottom of the table) – the standard is per 100g. However, if this is changed always ensure it is correct for the next food as it remains on what it last was – therefore it is easier to leave it as per 100g
  o Keep a hard copy of all added foods with their assigned food code on them

• Generating reports, getting and saving the results:
  o Click on the report tab
  o Change display options → click “design” → choose which tables/graphs are required from the content list – always include the nutrient table (provides total amount eaten and average daily intake (per day column) → save as
  o ALWAYS save a copy of the PDF (“save a copy” top left corner)
  o Click on the grid tab – provides a list of all the foods that have been entered as well as all the nutrient values for each food. Export this data to excel → click “export” and save as excel spreadsheet
SUB-STUDY PARTICIPANT INFORMATION SHEET

Sub-Study Title: Identification of genetic polymorphisms for vitamin D metabolism in Asian & Caucasian Women and analysis of the effects of vitamin D on DNA repair activity

You are invited to take part in a research study to identify the key genes associated with how our bodies use vitamin D (i.e. vitamin D metabolism). Before you decide whether you would like to participate, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please feel free to talk to others about the study if you wish.

Please do not hesitate to ask us if there is anything that is not clear or if you would like more information (contact Dr Laura Tripkovic, email: d2d3@surrey.ac.uk or tel: 01483 689222). Take time to decide whether or not you wish to take part.

1. What is the purpose of the study?
In order to understand further how vitamin D may benefit health, it is important to try to identify the key genes which are involved in vitamin D metabolism and to understand how it affects a range of different cellular processes.
As part of our nutrition study comparing the effects of vitamin D2 and vitamin D3 on health (The D2-D3 Study), we would like to assess women for their genetic profiles so that we can find out whether there are specific genes that affect how the body uses vitamin D. We would also like to compare genetic profiles of women from different ethnic backgrounds and age groups to see if these factors also affect how our bodies use vitamin D. Finally we want to follow up on some very recent evidence that vitamin D may act to enhance the capacity for cells in our bodies to repair damage to our DNA.

2. Why have I been chosen?
As a participant in the D2-D3 Study (main study), you have also been offered an opportunity to take part in this sub-study which aims to identify the key genes involved in vitamin D metabolism.

3. Do I have to take part?
No. It is entirely up to you to decide whether or not to take part. If you do, you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time and without giving a reason. A decision to withdraw at any time, or a decision not to take part, will not affect the standard of care you receive.

Declining to participate (or withdrawing your consent) in this Sub-Study will not affect your participation in the Main Study.

4. What will happen to me if I take part?
The procedures for the study will be exactly the same as those listed in the Main Study Participant Information Sheet. You will not be required to do anything further for this Sub-Study other than provide us with an additional 30ml of blood (equivalent to 6 teaspoons) at each study visit, which can be stored for assessment of the genes associated with vitamin D metabolism and the capacity of blood cells to repair DNA damage.
5. What do I have to do?
The activities and study visits that we ask you to complete for the D2-D3 Study are all detailed in the Main Study Participant Information Sheet. If you choose to take part in this additional Sub-Study then we only ask that you provide one extra blood sample (10ml) at each visit, alongside the other blood samples that will need to be taken for the Main Study.

6. What are the possible disadvantages and risks of taking part?
The risks of this Sub-Study to you are minimal. You may have some discomfort in your arm following the collection of the blood sample and some light bruising may occur. Occasionally some people can feel faint when they have their blood taken and so to help reduce the risk of this, you will have your blood sample taken either whilst you are lying down on a bed or reclined in a chair.

7. What are the possible benefits of taking part?
The purpose of this Sub-Study is to explore the possibility that there are specific genes in the body that affect how you use vitamin D. We believe that some people are more efficient than others in using vitamin D and this could be related to their genetic make-up. We also want to follow up on very recent findings suggestion that vitamin D may enhance DNA repair processes in our bodies.

As there is very little information currently available, the results will be unlikely to specifically help you at this time, however the data will help nutritional and genetic scientists understand vitamin D metabolism and bioactivity better. Using the information from this study and any future studies, the aim is to establish what would be the most efficient way to consume vitamin D in the diet that would benefit the population as a whole, irrespective of their genetic make up.

8. What happens when the research study stops?
At the end of the second study period (April 2013) the analysis of the collected data will commence. We will keep you regularly updated on the University of Surrey Vitamin D study website (details to follow) and with 6 monthly newsletters. Once all the genetic and DNA repair data have been collected and analysed, we will send you a report of our findings as a whole.

9. What will happen if I don’t want to carry on with the study?
You can withdraw from the study at any time. Any stored blood samples that can still be identified as yours will be destroyed if you so wish.

10. What if I have a concern about the study?
Any complaint about the way you have been dealt with during the study or any possible harm you might suffer will be addressed. Therefore if you have a concern about any aspect of this study, you should ask to speak with Professor Susan Lanham-New who is the Principal Investigator for this study (Tel No. 01483 686476; email: s.lanham-new@surrey.ac.uk).

11. Will my taking part in the study be kept confidential?
Yes. Your participation in this study will be kept confidential and you will be identified in the study with a unique study code. We would want to contact your GP to inform them that you are participating in our study.

12. What will happen to any samples I give?
The blood samples that you give and blood cells isolated from them will be frozen and stored in our secure -80°C freezer until the end of the study. Samples will then be measured for the D2-D3 Study Participant Information Sheet – Sub Study Version 1.4SS (08/05/2012) NHS REC Ref: 11/LO/0708
key genes associated with vitamin D metabolism and for DNA repair capacity. Any further measurements we wish to undertake will be submitted back to the Ethical Committee as an amendment and you will be kept informed by letter.

13. How long we propose to keep the samples?
We will keep the samples that you provide us in a secure -80°C freezer for a period of no less than 10 years.

14. Who is organising and funding the research?
The Nutrition and Bone Health team at the University of Surrey are the researchers undertaking this project. The Biotechnology and Biological Sciences Research Council’s Diet and Health Research Industry Club (BBSRC DRINC) is funding the project.

15. Who has reviewed the study?
This study has been reviewed and given a favourable ethical opinion from both the South-East Coast - Surrey NHS Research Ethics Committee and the University of Surrey Ethics Committee.

16. Contact Details
If you would like any further information about the study or to discuss any queries you may have, please contact Dr Laura Tripkovic on (01483) 689222 or d2d3@surrey.ac.uk. An answering machine will pick up any calls made out of office hours; you will then be contacted at the earliest opportunity.
D2-D3 Study Consent Form (Sub-Study)

Identification of genetic polymorphisms for vitamin D

Participant Number:

• I (name) ____________________________ voluntarily agree to take part in the above named research study conducted by the University of Surrey.

• I have read and understood the Sub-Study Participant Information Sheet provided (Date 08/05/2012; Version 1.4SS). I have been given a full explanation by the investigators of the nature and purpose of the study. I have been given the opportunity to ask questions on all aspects of the study and have understood the advice and information given as a result.

• I understand that all personal data is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998).

• I understand that I am free to withdraw from the study at any time without needing to justify my decision and without prejudice to me. I agree to my GP being informed of my participation in this study.

• I understand that in the event of my suffering a significant and enduring injury (including illness or disease) as a direct result of my participation in the study, compensation will be paid to me by the University of Surrey subject to certain provisos and limitations. The amount of compensation will be appropriate to the nature, severity and persistence of the injury, and will, in general terms, be consistent with the amount of damages commonly awarded for similar injury by an English court in cases where the liability has been admitted.

I confirm that I have read and understood the above and freely consent to participating in this study. I have been given adequate time to consider my participation and agree to comply with the instructions and restrictions of the study.

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Name of participant (BLOCK CAPITALS) Date Signature

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Name of witness (if appropriate) Date Signature

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Name of Researcher/person taking consent Date Signature
Appendix 39
SUB-STUDY PARTICIPANT CONSENT FORM

Study Title: Identification of genetic polymorphisms for vitamin D and bone health

CONSENT FORM

• I (name) __________________________________ ______ voluntarily agree to take part in the above named research study conducted by the University of Surrey.

• I have read and understood the Sub-Study Participant Information Sheet provided (Date 10th February 2006; Version SSIS1.1). I have been given a full explanation by the investigators of the nature and purpose of the study. I have been given the opportunity to ask questions on all aspects of the study and have understood the advice and information given as a result.

• I understand that all personal data is held and processed in the strictest confidence, and in accordance with the Data Protection Act (1998). I agree that I will not seek to restrict the use of the results of the study on the understanding that my anonymity is preserved.

• I understand that I am free to withdraw from the study at any time without needing to justify my decision and without prejudice to me. I agree to my GP being informed of my participation in this study

• I understand that the University of Surrey holds insurance, which covers claims for injury or deterioration in health, which arise directly from participation in clinical trials but that it applies only in those situations where the University can be shown to be legally liable.

• I confirm that I have read and understood the above and freely consent to participating in this study. I have been given adequate time to consider my participation and agree to comply with the instructions and restrictions of the study.

Name of volunteer (BLOCK CAPITALS) ________________________________
Signed
Address
Date

Name of witness (BLOCK CAPITALS) ________________________________
Signed
Date
DNA Extraction Protocol: Blood samples (1-10 ml)

1. Blood samples are vortexed in sample tubes and transferred to barcoded 50 ml tubes containing the appropriate volume (3x sample volume) of Binding Solution (Buffer B1). Lids are securely fastened and each tube is vortexed briefly.

2. Tubes are transferred to a shaker for 2 minutes to ensure that the sample and Buffer B1 are thoroughly mixed.

3. Tubes are incubated at room temperature for a minimum of 30 minutes to allow samples to lyse and bind.

4. Tubes are transferred to a shaker for 2 minutes and are subsequently centrifuged at 2500 x g for 2 minutes and visually inspected for pellet formation.

5. Working in a Class II cabinet, the supernatant is decanted from each tube ensuring that the pellet is not dislodged.

6. The appropriate volume of Buffer C1 is added to each tube, and all tubes are mixed for 30 seconds on the shaker. Tubes are centrifuged at 2500 x g for 2 minutes and the supernatant removed.

7. A second wash step with Buffer C1 is performed, as per Step 6.

8. Following removal of the supernatant, the appropriate volume of Buffer A1 is added to each tube. All tubes are mixed for 30 seconds on the shaker and subsequently centrifuged at 2500 x g for 2 minutes. Supernatant is removed.

9. A second wash step with Buffer A1 is performed, as per Step 8.

10. Following removal of the supernatant, the appropriate volume of Buffer W1 is added to each tube. All tubes are mixed for 30 seconds on the shaker and subsequently centrifuged at 2500 x g for 2 minutes. Supernatant is removed.

11. The appropriate volume of ethanol is added to each tube, and all tubes are mixed for 30 seconds on the shaker. Tubes are centrifuged at 2500 x g for 2 minutes and the supernatant removed. Tubes are laid upside down on absorbant tissue for at least 10 minutes (maximum 30 minutes).

12. Tubes are transferred to the oven (55°C) until pellets are completely dry.

13. The correct volume of Elution Buffer (E1) is added to each tube and tubes are transferred to a shaker for 2 minutes. Tubes are incubated at 55°C for 30 min. Tubes are removed from the oven and briefly mixed in the shaker.
14. Tubes are incubated at room temperature for 24 hours to ensure complete resuspension of the pellet. Tubes are then placed in a shaker for 2 minutes.

15. Tubes are transferred to the fridge for 1 hour, and subsequently centrifuged at 4°C at 2500 $\times$ g for 2 minutes.

16. Eluted DNA samples are then transferred to 96-well plates. Well G12 always contains the QC DNA sample and well H12 is usually left empty.

17. Barcoded plates are tracked to a -20°C freezer.

18. Thawed plates are briefly centrifuged. Using a height limiter plate and the FluidX system, samples are transferred to fresh 96-well plates, and normalised according to customer requirements. (Any dregs are retained in a separate plate).
The purpose of this document is to provide an explanation of how KASP™ genotyping chemistry works, and also to provide information on how data is collected and analysed in our service laboratory.

**KASP overview**

KASP genotyping assays are based on competitive allele-specific PCR and enable bi-allelic scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci. The SNP-specific KASP Assay mix and the universal KASP Master mix are added to DNA samples, a thermal cycling reaction is then performed, followed by an end-point fluorescent read. Bi-allelic discrimination is achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponds with two universal FRET (fluorescence resonant energy transfer) cassettes; one labelled with FAM™ dye and the other with HEX™ dye.

**Detailed explanation of KASP genotyping chemistry**

KASP™ genotyping assays are based on competitive allele-specific PCR and enable bi-allelic scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at specific loci. The SNP-specific KASP Assay mix and the universal KASP Master mix are added to DNA samples, a thermal cycling reaction is then performed, followed by an end-point fluorescent read. The KASP Assay mix contains three assay-specific non-labelled oligos: two allele-specific forward primers and one common reverse primer. The allele-specific primers each harbour a unique tail sequence that corresponds with a universal FRET (fluorescence resonant energy transfer) cassette; one labelled with FAM™ dye and the other with HEX™ dye. The KASP Master mix contains the universal FRET cassettes, ROX™ passive reference dye, taq polymerase, free nucleotides and MgCl₂ in an optimised buffer solution. During thermal cycling, the relevant allele-specific primer binds to the template and elongates, thus attaching the tail sequence to the newly synthesised strand. The complement of the allele-specific tail sequence is then generated during subsequent rounds of PCR, enabling the FRET cassette to bind to the DNA. The FRET cassette is no longer quenched and emits fluorescence. Bi-allelic discrimination is achieved through the competitive binding of the two allele-specific forward primers. If the genotype at a given SNP is homozygous, only one of the two possible fluorescent signals will be generated. If the genotype is heterozygous, a mixed fluorescent signal will be generated.
How KASP works

1) Assay components:
KASP uses three components: test DNA with the SNP of interest; KASP Assay mix containing two different, allele-specific, competing forward primers with unique tail sequences and one reverse primer; the KASP Master mix containing FRET cassette plus Taq polymerase in an optimised buffer solution.

2) Denatured template and annealing components – PCR round 1:

(allele-2 primer does not elongate)

(allele-1 primer binds and elongates)

In the first round of PCR, one of the allele-specific primers matches the target SNP and, with the common reverse primer, amplifies the target region.

3) Complement of allele-specific tail sequence generated – PCR round 2:

(Reverse primer binds, elongates and makes a complementary copy of the allele-1 tail.)

4) Signal generation – PCR round 3:

FAM-labelled oligo binds to new complementary tail sequence and is no longer quenched.

In further rounds of PCR, levels of allele-specific tail increase. The fluor labelled part of the FRET cassette is complementary to new tail sequences and binds, releasing the fluor from the quencher to generate a fluorescent signal.
Analysis of genotyping data in our service laboratory

All assays for human samples are tested on LGC Genomics’ in-house validation DNA prior to being run on customer samples. Assays are deemed to be working successfully if clusters are distinct and call rates are consistently high. If samples are non-human, the assay is validated on DNA supplied by the customer.

The data is automatically quality control checked on a per SNP basis. No template controls (NTCs) are included on each plate to enable the detection of contamination or non-specific amplification – these samples must not amplify during the reaction. The number of genotypes that are callable must be greater than 90% and minor allele frequency should be greater than 2% unless the SNP is known to be a very low frequency. A chi-squared value (X2) is also generated that assesses distribution in multinomial datasets based on the Hardy-Weinberg equation.

Following completion of the initial 35 cycles of PCR, all genotyping reaction plates run at LGC are read on a BMG PHERAStar plate reader. This initial read data is visually inspected by a member of the genotyping team to assess the progression of the PCR reaction. The plates are then recycled (3 cycles per recycle step) and read after each recycle step. The laboratory operator visually inspects the read data after each recycle step and, once they are satisfied that the PCR reaction has reached endpoint, indentifies plates as completed. At this stage, our in-house Kraken software will automatically call genotypes for your samples. Your project manager will access the plate read data in Kraken and perform a detailed analysis of the data. This may require them to correct the automatically called genotypes that Kraken has given. Version one of your genotyping results is then exported within the Kraken system. A second project manager will then second check these results, and verify or change them in collaboration with your project manager. The results are then ready to send to you, the customer.