TITLE PAGE

Title: Daily supplementation with 15µg of vitamin D_2 versus vitamin D_3 in raising wintertime 25-hydroxyvitamin D status in healthy South Asian and white European women: A 12-wk randomized, placebo-controlled, food fortification trial

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Disclaimers: N/A

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Sources of Support: This work was funded by the UK-based Biotechnology and Biological Sciences Research Council (BBSRC) as part of a BBSRC Diet and Health Research Industry Club (DRINC) grant - BB/I006192/1.

Awards: LT was recipient of a Young Investigator Award at the 2014 National Osteoporosis Society Conference (Birmingham, UK) for the vitamin D2 vs vitamin D3 findings. LW won the Post-Graduate Award at the 2016 Nutrition Society 75th Anniversary Conference (Dublin, Republic of Ireland) for vitamin D food fortification and the D2-D3 study findings

Short Running Head: The D2-D3 Study

Abbreviations: 25(OH)D, 25-hydroxyvitamin D;

Clinical Trial Registry No: This trial was registered with the ISRCTN trial registry at isrctn.com as ISRCTN23421591.

ABSTRACT

Background: There are conflicting views in the literature as to whether vitamin D_2 and vitamin D_3 are equally effective at raising and maintaining serum concentrations of 25-hydroxyvitamin [25(OH)D], particularly at lower doses of vitamin D.

Objective: We aimed to investigate whether vitamin D_2 or vitamin D_3 fortified in juice or food, at a relatively low dose of 15 µg/d, was effective in raising serum total 25(OH)D and to compare their respective efficacy in South Asian and white European women over the winter months, within the setting of a large randomized-controlled trial.

Design: A randomized, double-blind, placebo-controlled, food fortification trial was conducted in healthy South Asian and white European women aged 20-64 y (n = 335; Surrey, UK) who consumed either placebo, 15 µg vitamin D_2 juice, 15 µg vitamin D_2 biscuit, 15 µg vitamin D_3 juice or 15 µg vitamin D_3 biscuit daily for 12 wk. Serum 25(OH)D was measured by liquid-chromatography tandem mass spectrometry (LC/MS-MS) at baseline, week 6 and week 12 of the study.

Results: Post-intervention, in the two ethnic groups combined, both the D_3 biscuit and the D_3 juice groups demonstrated a significantly greater absolute incremental change (Δ) in total 25(OH)D when compared to the D_2 biscuit group (Δ 15.3nmol/l [95% CI 7.4, 23.3], $p<0.0003$ and Δ16.0nmol/l [95% CI 8.0, 23.9], $p<0.0001$), the D_2 juice group (Δ 16.3nmol/l [95% CI 8.4, 24.2], $p<0.0001$ and Δ 16.9nmol/l [95% CI 9.0, 24.8], $p<0.0001$), and the placebo group (Δ 42.3nmol/l [95% CI 34.4, 50.2], $p<0.0001$ and Δ 42.9nmol/l [95% CI 35.0, 50.8], $p<0.0002$).

Conclusions: Using a daily dose of vitamin D relevant to public health recommendations (15 µg) and in vehicles relevant to food fortification strategies, vitamin D_3 was more effective than vitamin D_2 in raising serum 25(OH)D in the wintertime. Vitamin D_3 may therefore be a preferential form to optimize vitamin D status within the general population.

Keywords: vitamin D, vitamin D_2, vitamin D_3, 25-hydroxyvitamin D, randomized controlled trial, food fortification, healthy women, South Asian, white European

INTRODUCTION

Historically, it has been suggested that there is no difference between vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol) in their effectiveness in improving vitamin D status (1-4). We and others have challenged this thinking (5), controversially (6). Over the past two decades, a number of trials have been completed comparing the relative efficacy of vitamin D_2 versus D_3 in raising serum total 25-hydroxyvitamin D (25(OH)D; the biological marker widely used to indicate vitamin D status), with mixed results. Whilst there is strong evidence that in large bolus doses vitamin D_3 is the more efficacious form (7-10), for lower doses the evidence is contradictory (11-13). From a meta-analysis published in 2012, it is clear that the studies have small cohort sizes and are consequently under-powered, and there is a large variation in the dosage and frequency of administration of vitamin D between studies (14). Hence, to date, no studies have been able to comprehensively answer two questions: 1) whether there is a significant difference in efficacy between vitamin D_2 and D_3 in raising total 25(OH)D, and if so, 2) whether the recommended daily allowance (RDA) of vitamin D in either form achieves and maintains a 25(OH)D concentration within an acceptable range for health?

Aside from the scientific interest in vitamin D, understanding and quantifying the comparative efficacy of vitamin D_2 and D_3 on total 25(OH)D is important to ensure that public health advice is as effective as possible in preventing vitamin D deficiency across the population. Current guidance given by the US National Institute of Health (NIH), the UK Department of Health, and other government bodies around the world, is that the two forms of vitamin D are equivalent and can be used to equal effect; although the NIH do acknowledge that vitamin D_3 offers greater efficacy when given in bolus doses.

In populations living at northerly latitudes, where there is an absence of UVB rays for endogenous vitamin D synthesis between the months of October to March alongside the

limited dietary sources of vitamin D, it is firmly established that vitamin D status is inadequate during the winter months (15-16). The diversity of ethnic backgrounds within such populations adds further complexity to the issue; Darling and colleagues have shown that in the UK those of South Asian origin were deficient (25(OH)D <30nmol/l) the entire year-round, irrespective of available dietary or UV sources of vitamin D (16).

Extending the use of vitamin D food fortification may be a key strategy in alleviating the risk of vitamin D deficiency within the population. However, given the current controversy surrounding the efficacy of vitamin D_2 and D_3, it is not yet clear whether either form may be the preferred option for food fortification in order to maximise the potential beneficial impact at a population-wide level.

The primary aim of the D2-D3 Study was to use a food-fortification model, designed to compare the efficacy of 15 μg/d (Institute of Medicine [IOM] RDA) of vitamin D_2 versus vitamin D_3 in raising serum total 25(OH)D in South Asian and white European women during the wintertime in the United Kingdom.

METHODS

Subjects

A total of 335 healthy, free living South Asian or white European women aged 20-64 y were recruited in this 12-wk food fortification intervention trial. Subjects were recruited in the Surrey (UK) area through the use of local contacts and advertisements, as well as through local GP surgeries with permission and support from the National Institute for Health Research Clinical Research Network (UKCRN ID 10695). The inclusion criteria ensured all participants were in good health, white European or South Asian (i.e. originating from India, Bangladesh, Pakistan or the Arabian Peninsula). Participants were also either pre-menopause, or >3 y post-menopause. Volunteers were excluded if they were unwilling to discontinue the consumption of vitamin D-containing supplements 4 wk before the initiation of the study and throughout the study. Volunteers were also excluded if they were regular sun-bed users or if they had been on a sunshine vacation within 4 wk before the initiation of the study, or planned to take a sunshine vacation during the 12 wk intervention. The exclusion criteria also included pregnancy and breastfeeding, malabsorption syndromes (i.e. coeliac disease), renal failure and any health conditions or use of medications that interfered with vitamin D metabolism or bone turnover.

Study design and randomization

This was a 12-wk double-blind, randomized, placebo-controlled, parallel food fortification trial based at the University of Surrey (UK). As described in Figure 1 (Consolidated Standards of Reporting Trials (CONSORT) flow diagram (17)), participants were allocated to one of five treatment groups: placebo juice with placebo biscuit (placebo); 15 µg vitamin D_2 juice with placebo biscuit (D2J); placebo juice with 15 µg vitamin D_2 biscuit (D2B); 15 µg

vitamin D_3 juice with placebo biscuit (D3J) and placebo juice with 15 µg vitamin D_3 biscuit (D3B).

Participants were allocated to a treatment group via a randomized allocation system using a computer-generated randomization programme generated by the trial statistician. The randomization 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 randomization). The trial statistician was responsible for keeping the code. The codes were shared with Campden BRI (Chipping Campden, UK) and the experimental intervention products were assigned the respective code during the packaging process by the manufacturers.

This D2-D3 Study took place over two consecutive winters (October 2011 to March 2012 and October 2012 to March 2013), to avoid interference of UV exposure on vitamin D status. The participants attended three face-to-face individual study appointments at the Clinical Investigation Unit (University of Surrey); one at the start of the trial (week 0), the middle (week 6) and the end (week 12). Participants were given intervention products (juice and biscuits) based on their randomization code at the start of the trial, and were requested to consume one juice and one biscuit per day for 12 wk. At all visits, a standardised set of anthropometrics were recorded (**Table 1**), in addition to a fasting blood sample to measure serum total 25(OH)D, $25(OH)D_2$, $25(OH)D_3$, calcium, albumin and parathyroid hormone (PTH) (Table 2). All blood samples were stored at -80°C prior to analysis. At the baseline and final visit participants were requested to complete a 4-day diet diary to assess dietary intakes, and wear a dosimeter (polysulphone badge) for seven days on their outer clothing to measure exposure to UV radiation.

Intervention Products

The intervention products were formulated and manufactured by Campden BRI (Chipping Campden, UK) (Juice (210g serving) 305.6 kJ, 0.2g fat, 0.9g protein, 17.6g carbohydrate, 17.2mg calcium; Biscuit (17g serving) 321.0 kJ, 3.6g fat, 1.0g protein, 10.6g carbohydrate, 15.6mg calcium) as either a placebo or were fortified with 15 µg of vitamin D_2 or vitamin D_3. Hemi-cellulose micro-encapsulated vitamin D_2 and D_3 (Lycored, Kent, UK) was added to the respective juice and biscuits during manufacture. High performance liquid chromatography tandem mass spectrophotometry (LC MS/MS) was used to determine the amount and stability of vitamin D_2 and D_3 in the orange juice and biscuits. The products were found to contain either no vitamin D_2 or D_3 (placebo) or vitamin D within 10% of their specified concentrations. Concentration of vitamin D_2 and D_3 was found to be stable after storage at room temperature for three months.

Laboratory Analysis

Serum 25(OH)D

Serum 25(OH)D, 25(OH)D_2 and 25(OH)D_3 concentrations were determined by LC-MS/MS using an AB Sciex 5500 tandem mass spectrophotometer (AB Sciex UK Ltd, Warrington, UK) and the MassChrom ® 25(OH)D_3/D_2 kit for LC-MS/MS (Chromsystems Instruments and Chemicals GmbH, Gräfelfing, Germany) following the manufacturers' instructions. Laboratory intra- and inter-assay CVs were 3.7% and 4.8% respectively. The Manchester laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient by the Vitamin D Quality Assurance Scheme (DEQAS).

Serum calcium, albumin and parathyroid hormone

Calcium, albumin and PTH concentrations were measured by Surrey Pathology Services (Frimley, Camberley, UK). Serum calcium was measured using an endpoint spectrophotometric reaction based on the o-cresolphthalein complexone (CPC) methodology, and serum albumin was measured using an endpoint spectrophotometric reaction based on the bromocresol green solution (BCG) dye binding methodology, both using the ADVIA 2400 Chemistry System (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK). Manufacturer's quoted inter- and intra-assay CVs for calcium were 1.9% and 1.1% respectively, and for albumin were 1.3% and 0.6% respectively. Serum calcium concentrations were adjusted for albumin concentrations. 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 were 3.4% and 4.0% respectively.

Assessment of dietary intakes, UV exposure and compliance

Dietary intakes were determined by inputting diet diary data (following a generic foods protocol) into the dietary analysis programme DietPlan6 (Forestfield Software Ltd, Horsham, UK), with standardised portion sizes obtained from the 'Food Portion Sizes' book (The Stationary Office, UK). UV exposure was measured by reading both pre- and post-intervention dosimeters at 330nm using a Cecil Aquarius CE7200 Double Beam Spectrophotometer (Cecil Instruments Ltd, Cambridge UK) to detect the change in absorbency. Results were then converted to Standard Erythemal Dose (SED) as previously described (16). Participant compliance to the study was assessed through a one-to-one interview with a researcher, and a packet count, at both week 6 and week 12. Regular

telephone contact (minimum fortnightly) assisted in encouraging and monitoring participant compliance through the duration of the study.

Ethical approval

This study received ethical approval from the South-East Coast (Surrey) National Health Service Research Ethics Committee (11/LO/0708) and the University of Surrey Ethics Committee (EC/2011/97/FHMS). All participants gave written informed consent in agreement with the Helsinki Declaration prior to commencing study activities; the full study protocol is available as a supplementary file.

Statistical analyses

The response of serum total 25(OH)D concentrations to vitamin D_2 or D_3 was the primary end-point, and formed the basis of the sample size calculations. A total of 320 subjects (white European *n* 240, South Asian *n* 80) at 90% power were required to: (i) detect a 0.6 SD effect size in serum 25(OH)D levels between placebo and 15µg in white European women for vitamin D_2 vs. vitamin D_3; (ii) detect a 1.1 SD effect size in serum 25(OH)D levels between placebo and 15µg in South Asian women for vitamin D_2 vs. vitamin D_3.

The biochemical data were analysed using SAS 9.2 (SAS Institute Inc, NC, USA), on the basis of intention-to-treat, and were analysed a) as non-transformed data to bring out increments relative to baseline (absolute and delta values) and b) as logarithmically-transformed data to bring out increments as percentage relative to baseline values. The data were then submitted to a general linear mixed model, using SAS PROC MIXED. Model independent variables were: baseline 25OHD status, age, BMI, ethnicity (white European and South Asian), time visit (the visits were: visit 1, for the model baseline covariate; visits 2 and

3, the two post-intervention visits). In the modelling, visit was a two-level (visits 2 and 3) repeated measure with unstructured variance-covariance matrix), intervention group (control group, D2 group and D3 group) and the following interactions – a) time visit by intervention group interaction; b) time visit by ethnicity interaction; c) ethnicity by intervention group interaction and d) time visit by ethnicity by intervention group interaction. Subject was a model random effect. The 'time visit' and 'subject' variables were modelled as random effects, the remaining independent variables were modelled as fixed effects.

In addition to including the above-mentioned four interaction terms as independent variables in our general linear mixed model, we tested the statistical significance of each of these interactions.

Missing data was treated in the modelling as being missing at random, with only the non-missing data being submitted to the general linear mixed model. The 95% confidence intervals and p values, involving contrasts adjusted for baseline, were used to obtain the statistical results quoted below, and were obtained using the ESTIMATE statement of SAS PROC MIXED, as well as the PDIFF option of the LSMEANS statement of SAS PROC MIXED. Contrast estimates for logarithmically-transformed data were expressed as percentage differences. We applied multiplicity correction to both the primary and secondary objectives using Bonferroni adjustment for a total of 18 p values; significance was therefore only accepted at $p<0.003$ [$p<0.05/18$]). We give details of the Bonferroni-adjusted significance throughout the results section. We did not apply the Bonferroni correction to the interaction testing. The data for $25(OH)D_2$ (and corrected calcium in certain instances) did not allow modelling of the non-logarithmically transformed data to be performed and thus this variable is only described as percentage (%) change relative to baseline, not absolute. For ease of comparison, $25(OH)D_2$, $25(OH)D_3$, parathyroid hormone and corrected calcium are presented as relative (%) change

relative to baseline (not absolute increments) within the text of the manuscript, with the geometric mean values presented in Table 3 also generated from the logarithmically transformed data.

RESULTS

Baseline participant characteristics

A total of 335 women were randomised and entered into the D2-D3 Study, forming five intervention groups. These are shown in **Table 1**. The study was carried out over two consecutive winter periods (Oct 2011-Mar 2012 and Oct 2012-Mar 2013) and participants were recruited between Oct-Jan 2012 and July-Jan 2013 respectively. As described in **Figure 1**, a total of 525 individuals were initially assessed for inclusion, with 190 deemed ineligible and 335 proceeding to join the study. Participant numbers (both ethnic groups combined) per intervention group were between *n* 65 to *n* 70. The numbers for the white European group in each randomisation category were between *n* 48 to *n* 51. The numbers for the South Asian group in each randomisation category were *n* 17 to *n* 19. The drop-out rate equated to 13.1% (*n* 44). However, all participants who commenced the study were included in the final analysis (Intention-To-Treat). We did not check for significant differences at baseline since the groups were randomly assigned and so any differences at baseline would have been explained by chance (**Tables 1-3**).

Significance testing for interactions

Results for the significance levels of the tests of interaction were as follows: The a) time visit x intervention group interaction term was significant for all the primary and secondary objective outcome measurements including total 25(OH)D status, PTH, $25(OH)D_2$ and $25(OH)D_3$ ($p<0.0004$ to $p<0.0001$ respectively). For total 25(OH)D status, there was a non-significant trend for b) time visit x ethnicity ($p<0.066$) but no significant differences for c) intervention group x ethnicity or d) time visit x intervention group x ethnicity. For PTH, b) time visit x ethnicity interaction was not significant and neither was c) intervention group x ethnicity. For d) time visit x intervention group x ethnicity interaction, this was significant

($p<0.04$). For $25(OH)D_2$, b) no significant interactions were found for time visit x ethnicity, but for c) a significant interaction was shown for intervention group x ethnicity ($p<0.001$), and for d) time visit x intervention group x ethnicity ($p<0.01$). Similar findings were found for $25(OH)D_3$: b) time visit x ethnicity interaction was significant ($p<0.0067$) and c) intervention group x ethnicity interaction was significant ($p<0.0001$) and d) a non-significant trend for time visit x intervention group x ethnicity interaction ($p<0.1$).

Total serum 25(OH)D concentrations in the two ethnic groups combined

As described in **Table 2**, the placebo group experienced a 25% reduction in total 25(OH)D over the 12-week intervention (Week 0: 44.8 nmol/l [95% CI 37.5, 52.1], Week 12: 33.5 nmol/l [95% CI 27.8, 39.3], Δ -11.2 nmol/l [95% CI -16.7, -5.8], ($p<0.0001$)).

When the data for the two ethnic groups were combined, both vitamin D_2 fortification products demonstrated a substantial impact upon total 25(OH)D concentrations, with a 33% and 34% increase over the course of the intervention for the D2J and D2B groups respectively. The vitamin D_3 fortification products demonstrated even greater effects, with the D3J and D3B groups increases in total 25(OH)D in the order of 75% and 74% respectively.

When comparing across intervention groups and considering change from baseline, the D3J group also demonstrated a significantly higher absolute change in total 25(OH)D concentrations over the course of the intervention when compared to D2J (Δ 16.9nmol/l [95% CI 9.0, 24.8], ($p<0.0005$), D2B (Δ16.0nmol/l [95% CI 8.0, 23.9], ($p<0.0003$) and placebo (Δ 42.9nmol/l [95% CI 35.0, 50.8], ($p<0.0005$). In addition, the D3B group demonstrated a significantly higher absolute change in total 25(OH)D when compared to the D2B group (Δ

15.3nmol/l [95% CI 7.4, 23.3], p<0.0003), the D2J group (Δ 16.3nmol/l [95% CI 8.4, 24.2], (*p*<0.0005), and the placebo group (Δ 42.3nmol/l [95% CI 34.4, 50.2], (*p*<0.0003).

No significant difference in absolute change between the D3J and D3B groups was detected over the time course of the intervention, thus indicating equivalent bioavailability (Δ 0.6nmol/l [95% CI -7.4, 8.6] (p<0.34). Similarly, for the D2J and D2B groups, no significant difference in absolute change for total 25(OH)D concentrations was detected between the two groups over the course of the intervention (Δ 0.9nmol/l [95% CI -6.9, 8.7], (p <0.25).

Since there were no significant interactions for ethnicity, we did not analyse further the 25OHD status for the Caucasian and South Asian groups separately. However we observed from the data (**Table 2**) that the South Asian women appeared to have a greater response to the vitamin D (both D2 and D3) compared to Caucasian women, likely due to their lower 25(OH)D status at baseline (<30nmol/l in all South Asian groups). We also observed that in those South Asian women in the vitamin D_2 group, 25OHD status did not reach 50nmol/l at the end of the 12 week period but those taking the vitamin D_3 juice did. When considering only those South Asian participants who completed the entire intervention (*n* 63, 71% completion), 72.7% of those South Asian women who consumed either vitamin D_3 product attained levels >50nmol/l whereas only 55.6% of SA participants consuming either D_2 product met the same serum 25(OH)D threshold. For the white European women who completed the study (*n* 228, 93% completion), all of those participants in the D3B and D3J groups achieved serum 25(OH)D levels >50nmol/l at the end of the intervention. In contrast, 90.9% of participants from the D2B and 89.4% from the D2J groups met the threshold of >50nmol/l post-intervention. When combining the D_2 groups, the attainment rate was

90.1%. For the placebo group, all SA women were below the 50nmol/l cut-off at the end of intervention, yet 42% of EU women were maintaining total 25(OH)D levels >50nmol/l.

Serum parathyroid hormone concentrations in two ethnic groups combined

Importantly, the parathyroid hormone (PTH) responded to the vitamin D in the direction expected physiologically (**Table 3**). Considering the percentage change from baseline, there were Bonferroni-corrected non-significant trends for reductions for the D2J, D3J and D3B groups ($p<0.03$), however there were no significant changes for the placebo and D2B groups. For corrected calcium (all groups), the post-intervention concentrations were significantly higher when compared relatively to the baseline ($p<0.0001$), however serum levels remained within the normal range expected clinically (**Table 3**).

Serum 25(OH)D_2 and 25(OH)D_3 concentrations in two ethnic groups combined

Given the fact that no significant differences were detected between the juice and biscuit groups within their respective vitamin D_2 and D_3 fortification strands, the groups' juice and biscuit data were aggregated to explore the response of 25(OH)D_2 and 25(OH)D_3 over the course of the intervention (taking into account the baseline values). As described in **Figure 2A**, for the aggregated vitamin D_2 intervention group (*n* 133), over the course of the intervention, there was a significant increase in 25(OH)D_2 compared to both the placebo (Estimated Percentage Difference [EPD] 2328.8% [95% CI 1717.4, 3113.7] ($p<0.0002$)) and D_3 groups (EPD 3018.7% [95% CI 2353.3, 3864.6] ($p<0.0002$)). For the 25(OH)D_3 response (**Figure 2B**), the aggregated D_3 intervention group (*n* 137) exhibited a significantly greater response over the course of the intervention when compared to the placebo (EPD 185.8% [95% CI 148.4, 228.7] ($p<0.0001$)) and D_2 groups (EPD 281.9% [95% CI 242.1, 326.3]

317 (p<0.0001)), however there was also a significant difference in 25(OH)D_3 responses between
318 the D_2 and placebo groups (EPD 33.6% [95% CI 16.2, 52.2] (p<0.0001)).
319
320 **Fortification product compliance**
321 There was a dropout rate of 13.1% (*n* 44) over the course of the study **(Figure 1)**, with a 71%
322 completion rate for the south Asian women and 93% completion rate for the white European
323 women (mean completion rate across the intervention groups per ethnicity). Reasons for drop-
324 out included dislike of food products/unwilling to comply (*n* 3), unable to tolerate products
325 with reports of nausea or heartburn (*n* 5), unable to obtain blood sample at mid-intervention or
326 final visit (*n* 6), change in family circumstances (*n* 7), moved from area *(n* 3), unwell during
327 trial and feeling unable to continue (*n* 3), and a number were lost to follow-up (*n* 17). The
328 participants who did complete the study demonstrated excellent compliance. On average,
329 participants consumed 94% of the products allocated to them, which translated into the
330 participants missing on average four biscuit and five juice portions over the course of the
331 intervention. The South Asian participants reported missing on average eight biscuit portions
332 and 11 juice portions, the white European participants missed an average of three biscuits and
333 four juice administrations.
334
335 **Dietary Intakes and UVB Exposure**
336 Dietary analysis confirmed the average intake of dietary vitamin D for the entire cohort at
337 baseline to be 2.7 ± 2.3µg per day (78.2% response rate, *n* 262). Mean intake for key nutrients
338 was as follows: Energy 7969.5 ± 1864.6kJ, Total Fat 78.6 ± 26.2g, Protein 72.8 ± 18.1g,
339 Carbohydrate 204.7 ± 51.7g and Calcium 849.1 ± 260.9mg.
340 Participants' UV exposure for the duration of the trial was minimal, with a mean exposure of
341 0.035 ± 0.039SED pre-intervention and 0.086 ± 0.137SED post-intervention for the cohort.

DISCUSSION

This study investigated whether vitamin D_2 or vitamin D_3 fortified in juice or food, at a relatively low dose of 15 μg/d, was effective in raising serum total 25(OH)D and compared the respective efficacy of these two forms of vitamin D in South Asian and white European women over the winter months. Whilst both vitamin D_2 and vitamin D_3 increased 25(OH)D status and prevented the decline in 25(OH) D status during the wintertime, the results showed that at a low, but relevant, dose of 15 μg/d, vitamin D_3 was more efficacious than vitamin D_2 at raising total 25(OH)D. This study is larger and more comprehensive than previous trials. We observed that although both vitamin D_2 and D_3 appeared to be effective in ensuring a sufficient vitamin D status for the white European participants (>50nmol/l) – e.g. 100% of European women who were in the vitamin D3 groups achieved serum 25(OH)D status >50nmol/l at the end of the 12 weeks, only ~90% of European women in the vitamin D2 groups achieved this level. By comparison, for the South Asian women, ~ 70% of women who were in the vitamin D3 groups achieved serum 25(OH)d status >50 nmol/l at the end of the study compared to ~50% of South Asian women who were in the vitamin D2 groups. The South Asian women commenced the study within deficiency status whereas the white European women commenced the study largely sufficient, thus when 25OHD status is in the deficient range, such as in South Asians in this study, it would be more efficacious to raise levels by using vitamin D_3 than vitamin D_2. Even this relatively low dose of fortification is effective and that use of large doses, as has been practice, to raise 25OHD, is not supported by these data.

It was also demonstrated that food fortification is not only an effective and highly acceptable method of conveying vitamin D to the population, but that acidic beverages such as juice (that also contain virtually no fat) are equally effective as a fortification vehicle when compared to more pH-stable, higher fat baked goods.

The tests for interaction between the time visit, intervention group and ethnicity showed some interesting findings: The time visit by intervention group interaction was significant across the board for the primary and secondary objectives. This was the main focus of the study – whether vitamin D_2 was different from vitamin D_3 with respect to changes in total 25(OH)D status and their concomitant differences from the placebo group . For total 25(OH)D status, where the interaction test involved ethnicity, the results were not significant, which was predictable given that our results showed no difference in the absolute rise in 25(OH)D status in response to fortification between white European and South Asian women. However, the statistical results/trends for the ethnicity interactions with respect to $25(OH)D_2$ and $25(OH)D_3$ status are intriguing and certainly warrant further investigation.

Our main findings, showing greater efficacy of vitamin D_3, is supported by a meta-analysis completed in 2012, which collated all studies to date that had directly compared the effects of vitamin D_2 and D_3 on total 25(OH)D (14). The meta-analysis indicated that vitamin D_3 was more efficacious than vitamin D_2 in raising total 25(OH)D. However the finding was mainly driven by studies using large single or intermittent bolus doses of vitamin D. Studies giving lower doses were largely unrepresentative, and the doses used (40-100 μg/d) were still higher than (a) global public health recommendations for daily consumption, and (b) intakes attainable without the use of supplements. Since the meta-analysis, there have been further randomized-controlled trials comparing vitamin D_2 and D_3 at lower daily doses (25-50 μg/d), although largely under-powered, that are consistent with our findings (18-20). Therefore this study strengthens the current evidence base, with provision of irrefutable data from a large cohort size following a robust study design.

An interesting result of our study is the response of the 25(OH)D metabolites; specifically the response of $25(OH)D_3$ to the vitamin D_2 intervention, and $25(OH)D_2$ to the vitamin D_3 intervention. The decrease in $25(OH)D_3$ that was shown in the aggregated vitamin D_2 group

(D2B and D2J combined; Figure 2) is consistent with previous findings from trials using daily doses of 25-100 μg/d (7, 19-21). Our study also showed a decrease in 25(OH)D_2 in the vitamin D_3 juice group, which has only previously been reported to have been found by Binkley and colleagues, although their data were not presented as too few participants had measureable 25(OH)D_2 at baseline (7). Whether this finding has not been shown in previous studies due to low concentrations of 25(OH)D_2 at baseline (typically <5 nmol/L) remains unclear, although Glendenning and colleagues found no change in 25(OH)D_2 in their vitamin D_3 group despite having higher baseline 25(OH)D_2 (13.3 nmol/L)(12).

A recent study by Oliveri and colleagues (22) took a pharmacokinetic approach to understand the mechanism behind the apparent difference in efficacy between vitamin D_2 and D_3. The group administered a loading dose (2,500 μg) at day 0, followed by two weeks of daily supplementation (120 μg/d, from day 7 to day 21) with either vitamin D_2 or D_3, and then a 56-day clearance period. Their data shows that at both the post-loading dose phase (day 7) and post-daily dosage phase (day 21) there is no significant difference between groups, although the D_3 group had higher concentrations of 25(OH)D; yet at end of the clearance phase, the D_3 group had significantly higher 25(OH)D than the D_2 group. Oliveri and colleagues calculated that the elimination half-life of 25(OH)D for the D_2 group was substantially shorter at 33 days when compared to 82 days for the D_3 group (22).

It is becoming clearer from both the literature and the results of this study, that there is a pronounced difference in the efficacy of vitamin D_2 and D_3 in raising total 25(OH)D. The mechanisms driving this differentiating factor appear to be focussed around the effect of vitamin D_2 on 25(OH)D_3, which indicates a possible mechanism encompassing competitive binding and differences in binding affinity between vitamin D_2 and D_3 with the vitamin D binding protein and hydroxylation enzymes. However, the shorter half-life of 25(OH)D_2 compared with 25(OH)D_3 (22-23) also suggests that the elimination or degradation of

25(OH)D is another mechanism explaining the differences in the efficacy. To further expand this field and develop the mechanism, the *in vivo* behaviour of the CYP2R1 and CYP27B1 enzymes must be understood.

One of the strengths of the current study is the relevance of the dose chosen - matching the RDA set by the IOM of 15 µg/d for those aged 0-65 y to maintain 25(OH)D concentrations >50 nmol/L (1) and the use of vitamin D-fortified foods instead of supplements. As there is a lack of natural dietary sources of vitamin D (typical vitamin D intakes are 2.8 µg/d within the UK (24)), and the use of supplements by individuals could be erratic and unreliable, food fortification may be an important option for improving vitamin D intakes across a population. In the UK, where the dietary recommended value (DRV) for vitamin D has recently increased from 0 to 10 µg/d (25-26), considerable media attention and discussion has been focused on how this DRV will be achieved (27-28). Therefore the use of the juice and biscuit were critical to demonstrate that if a food or beverage forms a habitual element of an individual's diet, this could prove an effective and consistent method of providing vitamin D. In order to calculate the most effective level of fortification for improving vitamin D status, further research and modelling of the impact of fortification strategies is necessary, particularly looking at a combination of fortified foods and/or forms of vitamin D, as opposed to single staple food items which have previously been considered in modelling approaches (29-30). The primary strength of the D2-D3 Study is the fact that it is a larger cohort than previous studies comparing vitamin D_2 to vitamin D_3, with very good compliance. The study was conducted during the winter months, thus eliminating the confounding influence of UV exposure. The measurement of 25(OH)D_2 and 25(OH)D_3 also provides additional information that is key to understanding the potential mechanism behind the observed difference in response to vitamin D_2 and D_3. When compared to other studies in

441 the field, additional strengths of the study lay in the use of extended stratification of the
442 intervention groups to ensure an equal spread of age, BMI and ethnicity.
443 Limitations of the study centre on the lack of opportunity to generate dose response data. The
444 provision of 15 µg/d of vitamin D_2 or D_3 as part of the study was appropriate given the current
445 IOM RDA for vitamin D but, ideally, additional streams of intervention groups would have
446 been implemented so that the same food fortification vehicles could be used but with differing
447 doses of vitamin D_2 and D_3 fortification. Dose response data would have provided valuable
448 insight into the physiological response to vitamin D and thus assisted in elucidating the
449 mechanism behind the observed differences seen in the current data.
450 Thus to extend the field of knowledge, future research should investigate the dose-response of
451 vitamin D_2 versus D_3 at levels attainable by the general population, i.e. 5-20 µg/d. Additional
452 analysis of vitamin D metabolites such as the vitamin D-binding protein and key
453 hydroxylation enzymes would provide a more detailed context in which to evaluate the
454 metabolism of vitamin D_2 and D_3.
455 In conclusion, the D2-D3 Study is the most robust randomized controlled trial to date, that
456 specifically compares the efficacy of a relatively low-dose vitamin D_2 and vitamin D_3 (15
457 ug/d; 600 IU/d) on total serum 25(OH)D status during the wintertime in both Caucasians and
458 South Asians. This study shows that vitamin D_3 is superior in raising total serum 25(OH)D
459 status when compared to vitamin D_2, and may be most helpful in persons where baseline
460 25(OH)D levels are below 50 nmol/L. However, both forms of vitamin D in fortified foods
461 are effective at raising total 25(OH)D and preventing vitamin D deficiency (as defined as a
462 25(OH)D status of <25nmol/l) during the wintertime.

Acknowledgements

The authors acknowledge the support of the National Institute of Health Research Clinical Research Network (NIHR CRN). The authors would like to thank the following parties for their great help and kind assistance in the identification, recruitment and retention of participants: Mrs Shahnaz Bano (Surrey County Council, UK), Mrs Fatima Bukhari and Mrs Rukhsana Hanjra (Islamic Resource Centre, Kingston, UK). The D2-D3 Study team are extremely grateful to Professor Peter Schroder and Mr James Phillips (BBSRC DRINC Programme), and Professors John Mathers (University of Newcastle) and Professor Hilary Powers (University of Sheffield) for their critical comments in the design of this study and its implementation. The authors are very grateful to Professor Derk-Jan Dijk (University of Surrey and Editor-in-Chief: Journal of Sleep Research) and Dr Kourosh Ahmadi (University of Surrey) who provided sound independent advice on the statistical interpretation of the data. Finally, the authors would like to formally acknowledge and give great appreciation for the robustness of the AJCN review process, and in particularly the statistical critique of the methods used and their subsequent interpretation.

Conflict of Interest

LT, LW, KH, SJ, SdL, CPS, GB, SP, GC, RE, EH and JB had no conflicts of interest to declare. SLN is Research Director for D3Tex Ltd which holds the UK Patent (with Gulf Corporation Council Patent Pending) for the use of any UVB material for the prevention of vitamin D deficiency in women who dress for cultural style.

This work was funded by the UK-based Biotechnology and Biological Sciences Research Council (BBSRC) as part of the BBSRC Diet and Health Research Industry Club (DRINC) grant: BB/I006192/1. The funder had no role in the design, implementation, analysis, or interpretation of the research.

488

489 **Authors' Contributions**

490 The authors' responsibilities were as follows (in author order) – KH, CS, GB, SP, GC, RE,
491 EH, JB and SLN designed research; LT, LW, KH, SdL and SLN conducted research; SP and
492 GC produced intervention products; LT, LW and JB managed samples and laboratory
493 analysis; LT, LW, SJ and SLN performed statistical analysis; LT, LW, SJ, KH and SLN wrote
494 the paper; SLN had primary responsibility for final content. All authors read and approved the
495 final manuscript.

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Table 1: Characteristics of participants at baseline, per intervention group

Baseline anthropometrics					
	Placebo (*n* 65)	**D2 Juice (*n* 67)**	**D2 Biscuit (*n* 66)**	**D3 Juice (*n* 70)**	**D3 Biscuit (*n* 67)**
Age (yrs)	44.1 ± 11.48	44.3 ± 11.18	43.2 ± 13.23	43.0 ± 12.73	43.7 ± 12.84
Height (m)	1.64 ± 0.07	1.64 ± 0.07	1.64 ± 0.06	1.65 ± 0.06	1.64 ± 0.07
Weight (kg)	65.8 ± 10.12	64.4 ± 8.30	64.8 ± 11.79	64.4 ± 10.28	63.6 ± 10.90
BMI (kg/m^2)	24.4 ± 3.62	24.2 ± 3.42	24.1 ± 4.45	23.8 ± 3.65	23.8 ± 3.82
Waist Circumference (cm)	82.9 ± 10.76	81.9 ± 9.93	81.9 ± 11.83	81.0 ± 11.68	82.1 ± 11.86
Waist:Hip Ratio	0.81 ± 0.08	0.81 ± 0.07	0.81 ± 0.07	0.79 ± 0.08	0.81 ± 0.08
Body fat (%)	30.1 ± 6.87	30.1 ± 5.54	30.5 ± 6.36	29.9 ± 6.75	29.3 ± 7.81
Systolic BP (mmHg)	118.9 ± 15.09	116.8 ± 14.78	120.0 ± 15.46	118.1 ± 12.69	117.4 ± 15.49
Diastolic BP (mmHg)	78.7 ± 9.69	77.5 ± 9.51	79.3 ± 9.48	77.9 ± 9.83	77.2 ± 10.27

Table 1: Data presented as mean ± SD.

Key: yrs – years; m – metre; kg – kilograms; BMI – Body mass index; kg/m^2 – kilograms per metre square; cm – centimetre; BP – Blood Pressure; mmHg – millimetres of mercury.

Table 2: Serum total 25-hydroxyvitamin D (25(OH)D) concentrations at baseline, 6 weeks and 12 weeks per intervention group

	Intervention Groups				
Total 25(OH)D (nmol/l)	**Placebo (*n* 65)**	**D2 Juice (*n* 67)**	**D2 Biscuit (*n* 66)**	**D3 Juice (*n* 70)**	**D3 Biscuit (*n* 67)**
Week 0 (baseline)					
All	44.8 (37.5, 52.1)	44.9 (37.8, 52.0)	46.1 (38.9, 53.4)	42.3 (35.4, 49.2)	41.9 (34.9, 48.9)
South Asian	*30.8 (18.3, 43.3)*	29.5 (17.3, 41.6)	30.5 (18.0, 42.9)	27.3 (15.5, 39.2)	20.5 (8.7, 32.3)
White European	*58.8 (51.4, 66.2)*	60.3 (52.9, 67.7)	61.8 (54.4, 69.1)	57.3 (50.1, 64.5)	63.4 (55.9, 70.8)
Week 6 (mid-intervention)					
All	36.2 (30.4, 41.9)*	58.7 (53.1, 64.4)*a	58.6 (52.9, 64.4)*b	69.0 (63.3, 74.8)*a	67.7 (61.9, 73.5)*b
South Asian	*23.2 (13.3, 33.1)*	45.7 (35.9, 55.5)	44.9 (34.9, 54.8)	54.3 (44.2, 64.4)	47.6 (37.6, 57.6)
White European	*49.2 (43.3, 55.0)*	71.7 (66.0, 77.4)	72.4 (66.6, 78.2)	83.7 (78.1, 89.3)	87.8 (82.0, 93.6)
Week 12 (end of trial)					
All	33.5 (27.8, 39.3)*	59.7 (53.9, 65.4)*a	61.9 (56.0, 67.7)*b	74.0 (68.1, 79.9)*a	73.0 (67.1, 78.9)*b
South Asian	*23.3 (13.3, 33.2)*	47.2 (37.2, 57.2)	48.6 (38.5, 58.6)	60.1 (49.7, 70.5)	53.2 (42.9, 63.4)
White European	*43.8 (38.0, 49.6)*	72.2 (66.5, 77.9)	75.2 (69.3, 81.0)	87.9 (82.3, 93.5)	92.8 (87.0, 98.6)

Table 2: Serum total 25(OH)D concentrations represented as mean (95%CI), sourced from non log-transformed data subjected to a general linear mixed model analysis.

n indicates the numbers of participants randomised to each intervention group, who were then analysed as part of an Intention-to-Treat analysis plan regardless of participation.

* indicates $p<0.0001$ for comparison between visit and baseline, within respective group (effect of time) for 'All' participants. *a* – significant difference between D2J and D3J for 'All' participants, $p\leq0.003$; *b* – significant difference between D2B and D3B for 'All' participants, $p\leq0.002$; Results for the significance levels of the tests of interaction were as follows: The a) time visit x group interaction term was significant for the primary objective outcome measurements of total 25(OH)D ($p<0.0004$). For total 25(OH)D status, there was a non-significant trend for b) time visit x ethnicity ($p<0.066$) but no significant differences for c) intervention group x ethnicity or d) time visit x intervention group x ethnicity. Model independent variables were: baseline 25OHD status, age, BMI, ethnicity, time visit, intervention group and the following interactions – a) time visit by intervention group interaction; b) time visit by ethnicity interaction; c) ethnicity by intervention group interaction and d) time visit by ethnicity by intervention group interaction.

Table 3: Serum 25(OH)D_2, 25(OH)D_3, calcium and parathyroid hormone (PTH) concentrations at baseline, 6 weeks and 12 weeks per intervention group

	Intervention Groups				
	Placebo (*n* 65)	**D2 Juice (*n* 67)**	**D2 Biscuit (*n* 66)**	**D3 Juice (*n* 70)**	**D3 Biscuit (*n* 67)**
Week 0 (baseline)					
25(OH)D_2 (nmol/l) *25(OH)D_3 (nmol/l)*	*1.38 (1.05, 1.82)* *32.1 (27.5, 37.6)*	*0.97 (0.74, 1.27)* *33.7 (28.9, 39.3)*	*1.23 (0.94, 1.62)* *33.7 (28.8, 39.4)*	*1.14 (0.88, 1.48)* *30.9 (26.6, 35.9)*	*1.14 (0.87, 1.49)* *29.4 (25.3, 34.2)*
Adj. Calcium (mmol/l)	2.23 (2.21, 2.25)	2.24 (2.23, 2.26)	2.25 (2.23, 2.27)	2.25 (2.23, 2.27)	2.23 (2.22, 2.25)
PTH (pmol/l)	4.99 (4.48, 5.57)	5.17 (4.64, 5.75)	4.89 (4.38, 5.45)	4.85 (4.37, 5.38)	5.01 (4.51, 5.57)
Week 6 (mid-intervention)					
25(OH)D_2 (nmol/l) *25(OH)D_3 (nmol/l)*	*1.37 (1.10, 1.71)* *25.4 (22.3, 28.9)**	*28.60 (22.99, 35.57)*^{*a} *20.8 (18.3, 23.6)*^{*a}	*26.59 (21.30, 33.20)*^{*b} *22.7 (19.9, 25.8)*^{*b}	*0.82 (0.66, 1.03)*^{*a} *61.4 (54.0, 69.8)*^{*a}	*1.07 (0.85, 1.35)*^{b} *61.5 (54.0, 69.9)*^{*b}
Adj. Calcium (mmol/l)	2.20 (2.18, 2.22)*	2.19 (2.17, 2.21)*	2.19 (2.18, 2.21)*	2.20 (2.18, 2.22)*	2.19 (2.17, 2.21)*
PTH (pmol/l)	4.96 (4.45, 5.52)	4.93 (4.43, 5.48)	4.94 (4.43, 5.51)	4.58 (4.10, 5.12)	4.77 (4.27, 5.33)
Week 12 (end of trial)					
25(OH)D_2 (nmol/l) *25(OH)D_3 (nmol/l)*	*1.59 (1.25, 2.02)* *24.3 (21.2, 27.7)**	*29.54 (23.21, 37.58)*^{*a} *17.0 (14.9, 19.5)*^{*a}	*31.27 (24.56, 39.82)*^{*b} *21.4 (18.7, 24.5)*^{*b}	*0.89 (0.69, 1.14)*^{*a} *65.4 (57.1, 74.9)*$^{* a}$	*1.17 (0.91, 1.51)*^{b} *64.9 (56.6, 74.3)*^{*b}
Adj. Calcium (mmol/l)	2.28 (2.26, 2.31)*	2.30 (2.28, 2.32)*	2.29 (2.27, 2.31)*	2.29 (2.27, 2.32)*	2.29 (2.27, 2.32)*
PTH (pmol/l)	5.27 (4.69, 5.91)	4.65 (4.14, 5.22)*	4.73 (4.21, 5.31)	3.98 (3.54, 4.49)*	4.13 (3.66, 4.66)*

Table 3: Vitamin D metabolites, parathyroid hormone and corrected calcium concentrations represented as geometric mean (95%CI), sourced from logarithmically-transformed data subjected to a general linear mixed model analysis. *n* indicates the numbers of participants randomised to each intervention group, who were then analysed as part of an Intention-to-Treat model at the end of the trial, regardless of participation. * indicates p<0.001 for comparison between visit and baseline, within respective group (effect of time). *a* – significant difference between D2J and D3J, p<0.002; *b* – significant difference between D2B and D3B, p<0.003. Results for the significance levels of the tests of interaction were as follows: The a) time visit x group interaction term was significant for all the secondary objective outcome measurements including total 25(OH)D, PTH, 25(OH)D_2 and 25(OH)D_3 (p<0.0004

to $p<0.0001$ respectively). For PTH, b) time visit x ethnicity interaction was not significant and neither was c) intervention group x ethnicity. For d) time visit x intervention group x ethnicity interaction, this was significant ($p<0.04$). For $25(OH)D_2$, b) no significant interactions were found for time visit x ethnicity, but for c) a significant interaction was shown for intervention group x ethnicity ($p<0.001$), and for d) time visit x intervention group x ethnicity ($p<0.01$). Similar findings were found for $25(OH)D_3$: b) time visit x ethnicity interaction was significant ($p<0.0067$) and c) intervention group x ethnicity interaction was significant ($p<0.0001$) and d) a non-significant trend for time visit x intervention group x ethnicity interaction ($p<0.1$).

Key: Adj. Calcium – Serum calcium concentration adjusted for concomitant albumin level, using the formula [(40 - albumin) x 0.02] + Calcium. PTH – Parathyroid Hormone.

Figure legends:

Figure 1: Consolidated Standards of Reporting Trials (CONSORT) flow diagram indicating the number of participants screened, recruited, randomized and analysed as part of the D2D3 Study.

Figure 2: Vitamin D metabolite responses per aggregated intervention group. Geometric mean (95%CI) serum concentrations per time point are shown, sourced from log-transformed data subjected to a general linear mixed model analysis (Intention-to-treat). (A) 25(OH)D_2. (B) 25(OH)D_3. Placebo group *n* 65, D_2 group *n* 133, D_3 group *n* 137. *a* – significant difference between placebo and D2 group over the intervention period, $p<0.0005$; *b* - significant difference between placebo and D3 group over the intervention period, $p<0.0005$; *c* - significant difference between D2 and D3 group over the intervention period, $p<0.003$.

Key: D_3 aggregated intervention group; D_2 aggregated intervention group; Placebo group

Figure 1

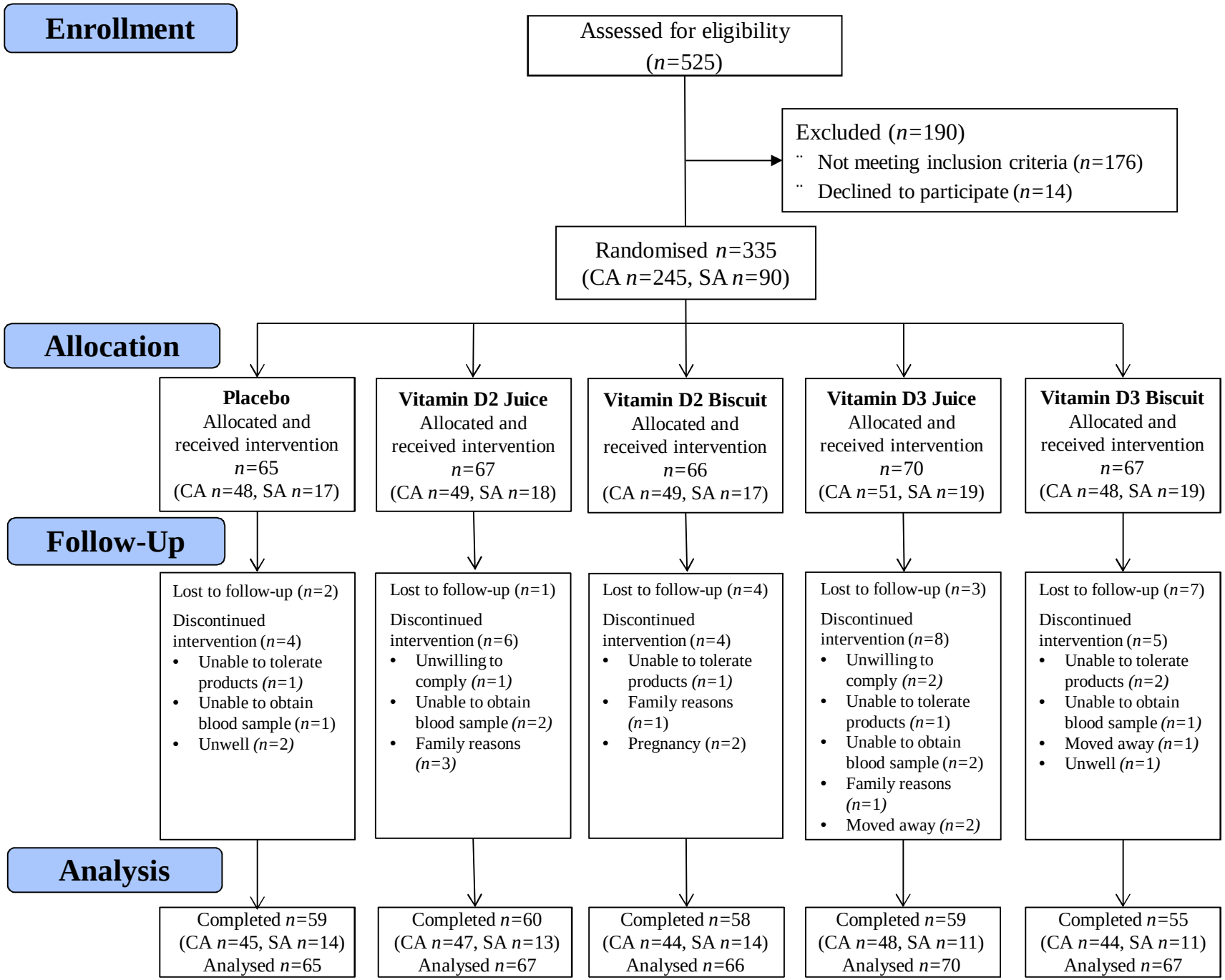

Enrollment
Assessed for eligibility (n=525)
Excluded (n=190)
¨ Not meeting inclusion criteria (n=176)
¨ Declined to participate (n=14)
Randomised n=335 (CA n=245, SA n=90)
Allocation
Placebo Allocated and received intervention n=65 (CA n=48, SA n=17)
Vitamin D2 Juice Allocated and received intervention n=67 (CA n=49, SA n=18)
Vitamin D2 Biscuit Allocated and received intervention n=66 (CA n=49, SA n=17)
Vitamin D3 Juice Allocated and received intervention n=70 (CA n=51, SA n=19)
Vitamin D3 Biscuit Allocated and received intervention n=67 (CA n=48, SA n=19)
Follow-Up
Lost to follow-up (n=2)
Discontinued intervention (n=4)
• Unable to tolerate products (n=1)
• Unable to obtain blood sample (n=1)
• Unwell (n=2)
Lost to follow-up (n=1)
Discontinued intervention (n=6)
• Unwilling to comply (n=1)
• Unable to obtain blood sample (n=2)
• Family reasons (n=3)
Lost to follow-up (n=4)
Discontinued intervention (n=4)
• Unable to tolerate products (n=1)
• Family reasons (n=1)
• Pregnancy (n=2)
Lost to follow-up (n=3)
Discontinued intervention (n=8)
• Unwilling to comply (n=2)
• Unable to tolerate products (n=1)
• Unable to obtain blood sample (n=2)
• Family reasons (n=1)
• Moved away (n=2)
Lost to follow-up (n=7)
Discontinued intervention (n=5)
• Unable to tolerate products (n=2)
• Unable to obtain blood sample (n=1)
• Moved away (n=1)
• Unwell (n=1)
Analysis
Completed n=59 (CA n=45, SA n=14) Analysed n=65
Completed n=60 (CA n=47, SA n=13) Analysed n=67
Completed n=58 (CA n=44, SA n=14) Analysed n=66
Completed n=59 (CA n=48, SA n=11) Analysed n=70
Completed n=55 (CA n=44, SA n=11) Analysed n=67

Figure 2

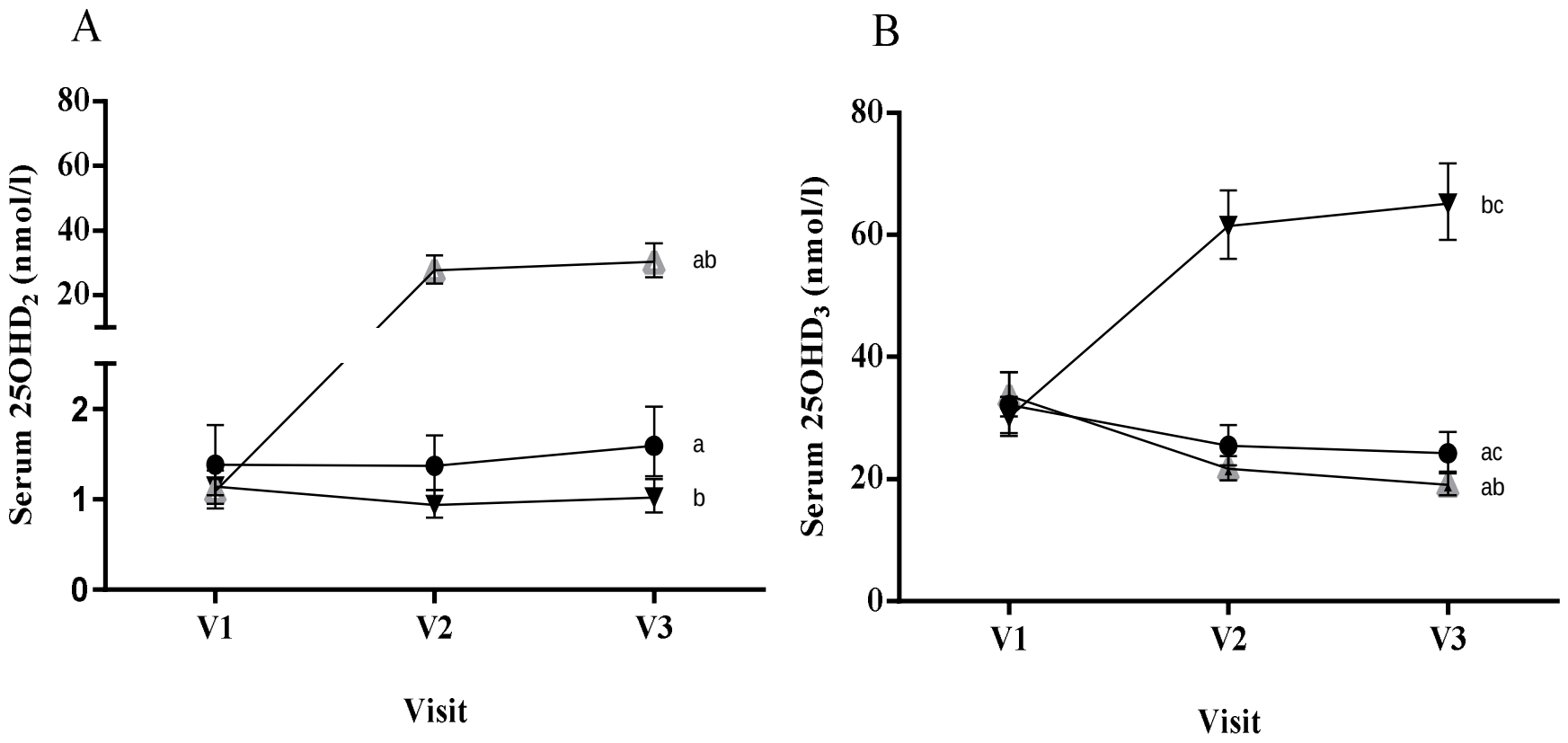
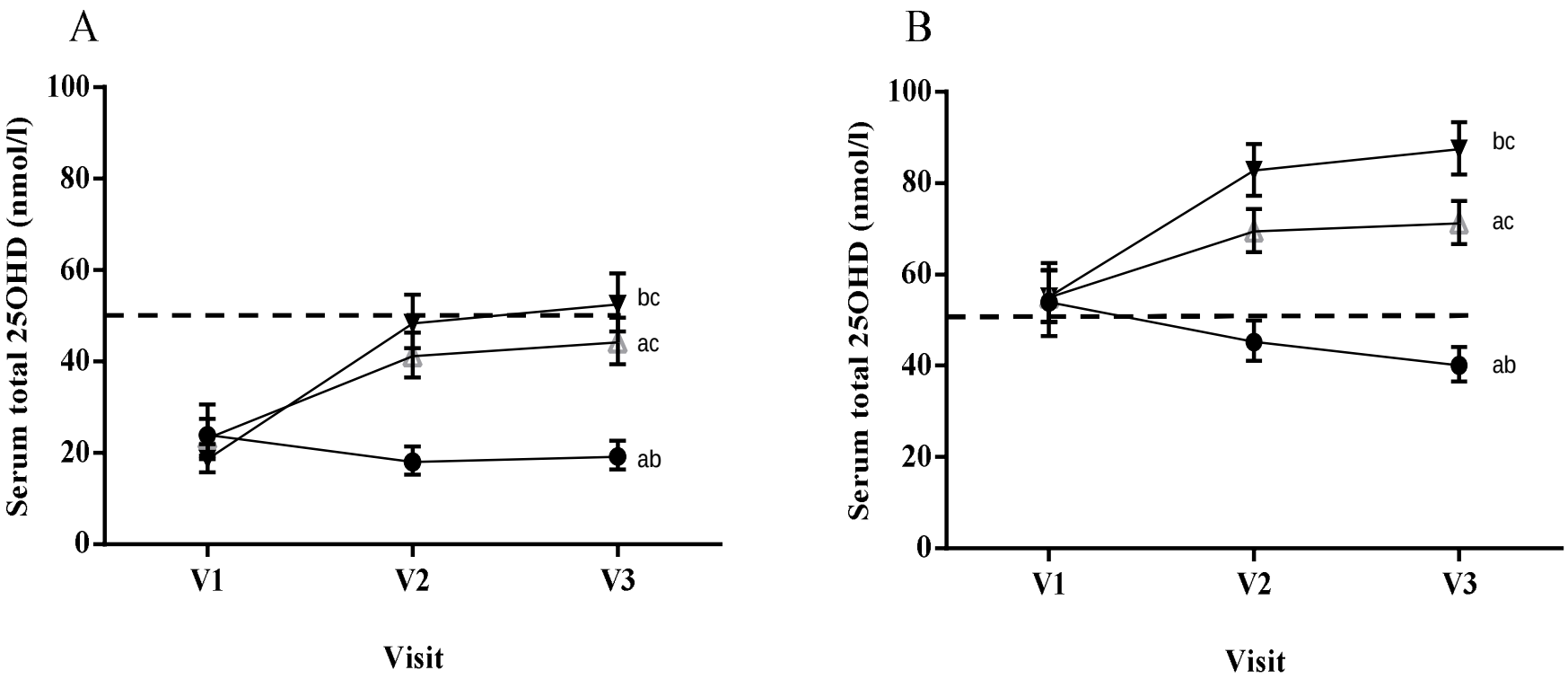

A
80
60
40
20
2
1
0
Serum 25OHD2 (nmol/l)
ab
a
b
V1
V2
V3
Visit
B
80
60
40
20
0
Serum 25OHD3 (nmol/l)
bc
ac
ab
V1
V2
V3
Visit

Figure 3


A
B
Serum total 25OHD (nmol/l)
100
80
60
40
20
0
V1
V2
V3
Visit
bc
ac
ab