

TITLE PAGE

Title: Daily supplementation with 15µg of vitamin D₂ versus vitamin D₃ 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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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.

1 ABSTRACT

2 **Background:** There are conflicting views in the literature as to whether vitamin D₂ and
3 vitamin D₃ are equally effective at raising and maintaining serum concentrations of 25-
4 hydroxyvitamin [25(OH)D], particularly at lower doses of vitamin D.

5 **Objective:** We aimed to investigate whether vitamin D₂ or vitamin D₃ fortified in juice or
6 food, at a relatively low dose of 15 µg/d, was effective in raising serum total 25(OH)D and to
7 compare their respective efficacy in South Asian and white European women over the winter
8 months, within the setting of a large randomized-controlled trial.

9 **Design:** A randomized, double-blind, placebo-controlled, food fortification trial was
10 conducted in healthy South Asian and white European women aged 20-64 y (n = 335; Surrey,
11 UK) who consumed either placebo, 15 µg vitamin D₂ juice, 15 µg vitamin D₂ biscuit, 15 µg
12 vitamin D₃ juice or 15 µg vitamin D₃ biscuit daily for 12 wk. Serum 25(OH)D was measured
13 by liquid-chromatography tandem mass spectrometry (LC/MS-MS) at baseline, week 6 and
14 week 12 of the study.

15 **Results:** Post-intervention, in the two ethnic groups combined, both the D₃ biscuit and the D₃
16 juice groups demonstrated a significantly greater absolute incremental change (Δ) in total
17 25(OH)D when compared to the D₂ biscuit group (Δ 15.3nmol/l [95% CI 7.4, 23.3], p<0.0003
18 and Δ16.0nmol/l [95% CI 8.0, 23.9], p<0.0001), the D₂ juice group (Δ 16.3nmol/l [95% CI
19 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
20 (Δ 42.3nmol/l [95% CI 34.4, 50.2], p<0.0001 and Δ 42.9nmol/l [95% CI 35.0, 50.8],
21 p<0.0002).

22 **Conclusions:** Using a daily dose of vitamin D relevant to public health recommendations (15
23 µg) and in vehicles relevant to food fortification strategies, vitamin D₃ was more effective
24 than vitamin D₂ in raising serum 25(OH)D in the wintertime. Vitamin D₃ may therefore be a
25 preferential form to optimize vitamin D status within the general population.

26 **Keywords:** vitamin D, vitamin D₂, vitamin D₃, 25-hydroxyvitamin D, randomized controlled
27 trial, food fortification, healthy women, South Asian, white European

28 INTRODUCTION

29 Historically, it has been suggested that there is no difference between vitamin D₂
30 (ergocalciferol) and vitamin D₃ (cholecalciferol) in their effectiveness in improving vitamin D
31 status (1-4). We and others have challenged this thinking (5), controversially (6). Over the
32 past two decades, a number of trials have been completed comparing the relative efficacy of
33 vitamin D₂ versus D₃ in raising serum total 25-hydroxyvitamin D (25(OH)D; the biological
34 marker widely used to indicate vitamin D status), with mixed results. Whilst there is strong
35 evidence that in large bolus doses vitamin D₃ is the more efficacious form (7-10), for lower
36 doses the evidence is contradictory (11-13). From a meta-analysis published in 2012, it is
37 clear that the studies have small cohort sizes and are consequently under-powered, and there
38 is a large variation in the dosage and frequency of administration of vitamin D between
39 studies (14). Hence, to date, no studies have been able to comprehensively answer two
40 questions: 1) whether there is a significant difference in efficacy between vitamin D₂ and D₃
41 in raising total 25(OH)D, and if so, 2) whether the recommended daily allowance (RDA) of
42 vitamin D in either form achieves and maintains a 25(OH)D concentration within an
43 acceptable range for health?

44 Aside from the scientific interest in vitamin D, understanding and quantifying the
45 comparative efficacy of vitamin D₂ and D₃ on total 25(OH)D is important to ensure that
46 public health advice is as effective as possible in preventing vitamin D deficiency across the
47 population. Current guidance given by the US National Institute of Health (NIH), the UK
48 Department of Health, and other government bodies around the world, is that the two forms of
49 vitamin D are equivalent and can be used to equal effect; although the NIH do acknowledge
50 that vitamin D₃ offers greater efficacy when given in bolus doses.

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

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

58 Extending the use of vitamin D food fortification may be a key strategy in alleviating the risk
59 of vitamin D deficiency within the population. However, given the current controversy
60 surrounding the efficacy of vitamin D₂ and D₃, it is not yet clear whether either form may be
61 the preferred option for food fortification in order to maximise the potential beneficial impact
62 at a population-wide level.

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

67 **METHODS**

68 **Subjects**

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

84

85 **Study design and randomization**

86 This was a 12-wk double-blind, randomized, placebo-controlled, parallel food fortification
87 trial based at the University of Surrey (UK). As described in Figure 1 (Consolidated
88 Standards of Reporting Trials (CONSORT) flow diagram (17)), participants were allocated to
89 one of five treatment groups: placebo juice with placebo biscuit (placebo); 15 µg vitamin D₂
90 juice with placebo biscuit (D2J); placebo juice with 15 µg vitamin D₂ biscuit (D2B); 15 µg

91 vitamin D₃ juice with placebo biscuit (D3J) and placebo juice with 15 µg vitamin D₃ biscuit
92 (D3B).

93
94 Participants were allocated to a treatment group via a randomized allocation system using a
95 computer-generated randomization programme generated by the trial statistician. The
96 randomization was stratified to take into account the participants' ethnicity, BMI and age, and
97 was verified by the trial statistician with the codes assigned to the participants by a trial
98 investigator (the investigator was blinded to the randomization). The trial statistician was
99 responsible for keeping the code. The codes were shared with Campden BRI (Chipping
100 Campden, UK) and the experimental intervention products were assigned the respective code
101 during the packaging process by the manufacturers.

102
103 This D2-D3 Study took place over two consecutive winters (October 2011 to March 2012 and
104 October 2012 to March 2013), to avoid interference of UV exposure on vitamin D status. The
105 participants attended three face-to-face individual study appointments at the Clinical
106 Investigation Unit (University of Surrey); one at the start of the trial (week 0), the middle
107 (week 6) and the end (week 12). Participants were given intervention products (juice and
108 biscuits) based on their randomization code at the start of the trial, and were requested to
109 consume one juice and one biscuit per day for 12 wk. At all visits, a standardised set of
110 anthropometrics were recorded (**Table 1**), in addition to a fasting blood sample to measure
111 serum total 25(OH)D, 25(OH)D₂, 25(OH)D₃, calcium, albumin and parathyroid hormone
112 (PTH) (Table 2). All blood samples were stored at -80°C prior to analysis. At the baseline and
113 final visit participants were requested to complete a 4-day diet diary to assess dietary intakes,
114 and wear a dosimeter (polysulphone badge) for seven days on their outer clothing to measure
115 exposure to UV radiation.

116 Intervention Products

117 The intervention products were formulated and manufactured by Campden BRI (Chipping
118 Campden, UK) (Juice (210g serving) 305.6 kJ, 0.2g fat, 0.9g protein, 17.6g carbohydrate,
119 17.2mg calcium; Biscuit (17g serving) 321.0 kJ, 3.6g fat, 1.0g protein, 10.6g carbohydrate,
120 15.6mg calcium) as either a placebo or were fortified with 15 µg of vitamin D₂ or vitamin D₃.
121 Hemi-cellulose micro-encapsulated vitamin D₂ and D₃ (Lycored, Kent, UK) was added to the
122 respective juice and biscuits during manufacture. High performance liquid chromatography
123 tandem mass spectrophotometry (LC MS/MS) was used to determine the amount and stability
124 of vitamin D₂ and D₃ in the orange juice and biscuits. The products were found to contain
125 either no vitamin D₂ or D₃ (placebo) or vitamin D within 10% of their specified
126 concentrations. Concentration of vitamin D₂ and D₃ was found to be stable after storage at
127 room temperature for three months.

128

129 Laboratory Analysis*130 Serum 25(OH)D*

131 Serum 25(OH)D, 25(OH)D₂ and 25(OH)D₃ concentrations were determined by LC-MS/MS
132 using an AB Sciex 5500 tandem mass spectrophotometer (AB Sciex UK Ltd, Warrington,
133 UK) and the MassChrom ® 25(OH)D₃/D₂ kit for LC-MS/MS (Chromsystems Instruments and
134 Chemicals GmbH, Gräfelfing, Germany) following the manufacturers' instructions.
135 Laboratory intra- and inter-assay CVs were 3.7% and 4.8% respectively. The Manchester
136 laboratory is accredited by CPA UK (CPA number 0865) and has been certified as proficient
137 by the Vitamin D Quality Assurance Scheme (DEQAS).

138

139

140

141 *Serum calcium, albumin and parathyroid hormone*

142 Calcium, albumin and PTH concentrations were measured by Surrey Pathology Services
143 (Frimley, Camberley, UK). Serum calcium was measured using an endpoint
144 spectrophotometric reaction based on the o-cresolphthalein complexone (CPC) methodology,
145 and serum albumin was measured using an endpoint spectrophotometric reaction based on the
146 bromocresol green solution (BCG) dye binding methodology, both using the ADVIA 2400
147 Chemistry System (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK).
148 Manufacturer's quoted inter- and intra-assay CVs for calcium were 1.9% and 1.1%
149 respectively, and for albumin were 1.3% and 0.6% respectively. Serum calcium
150 concentrations were adjusted for albumin concentrations. Plasma intact PTH was measured
151 using a two-site sandwich chemiluminescent immunoassay using the ADVIA Centaur XP
152 Immunoassay System (Siemens Healthcare Diagnostics Ltd, Frimley, Camberley, UK).
153 Manufacturer's quoted inter- and intra-assay CVs were 3.4% and 4.0% respectively.

154

155 *Assessment of dietary intakes, UV exposure and compliance*

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

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

167

168 **Ethical approval**

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

174

175 **Statistical analyses**

176 The response of serum total 25(OH)D concentrations to vitamin D₂ or D₃ was the primary
177 end-point, and formed the basis of the sample size calculations. A total of 320 subjects (white
178 European *n* 240, South Asian *n* 80) at 90% power were required to: (i) detect a 0.6 SD effect
179 size in serum 25(OH)D levels between placebo and 15µg in white European women for
180 vitamin D₂ vs. vitamin D₃; (ii) detect a 1.1 SD effect size in serum 25(OH)D levels between
181 placebo and 15µg in South Asian women for vitamin D₂ vs. vitamin D₃.

182

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

190 3, the two post-intervention visits). In the modelling, visit was a two-level (visits 2 and 3)
191 repeated measure with unstructured variance-covariance matrix), intervention group (control
192 group, D2 group and D3 group) and the following interactions – a) time visit by intervention
193 group interaction; b) time visit by ethnicity interaction; c) ethnicity by intervention group
194 interaction and d) time visit by ethnicity by intervention group interaction. Subject was a
195 model random effect. The ‘time visit’ and ‘subject’ variables were modelled as random
196 effects, the remaining independent variables were modelled as fixed effects.

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

200
201 Missing data was treated in the modelling as being missing at random, with only the non-
202 missing data being submitted to the general linear mixed model. The 95% confidence intervals
203 and p values, involving contrasts adjusted for baseline, were used to obtain the statistical results
204 quoted below, and were obtained using the ESTIMATE statement of SAS PROC MIXED, as
205 well as the PDIFF option of the LSMEANS statement of SAS PROC MIXED. Contrast
206 estimates for logarithmically-transformed data were expressed as percentage differences. We
207 applied multiplicity correction to both the primary and secondary objectives using Bonferroni
208 adjustment for a total of 18 p values; significance was therefore only accepted at $p < 0.003$
209 [$p < 0.05/18$]. We give details of the Bonferroni-adjusted significance throughout the results
210 section. We did not apply the Bonferroni correction to the interaction testing. The data for
211 25(OH)D₂ (and corrected calcium in certain instances) did not allow modelling of the non-
212 logarithmically transformed data to be performed and thus this variable is only described as
213 percentage (%) change relative to baseline, not absolute. For ease of comparison, 25(OH)D₂,
214 25(OH)D₃, parathyroid hormone and corrected calcium are presented as relative (%) change

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

218

219

220 RESULTS

221 Baseline participant characteristics

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

235

236 Significance testing for interactions

237 Results for the significance levels of the tests of interaction were as follows: The a) time visit
238 x intervention group interaction term was significant for all the primary and secondary
239 objective outcome measurements including total 25(OH)D status, PTH, 25(OH)D₂ and
240 25(OH)D₃ ($p < 0.0004$ to $p < 0.0001$ respectively). For total 25(OH)D status, there was a non-
241 significant trend for b) time visit x ethnicity ($p < 0.066$) but no significant differences for c)
242 intervention group x ethnicity or d) time visit x intervention group x ethnicity. For PTH, b)
243 time visit x ethnicity interaction was not significant and neither was c) intervention group x
244 ethnicity. For d) time visit x intervention group x ethnicity interaction, this was significant

245 ($p < 0.04$). For 25(OH)D₂, b) no significant interactions were found for time visit x ethnicity,
246 but for c) a significant interaction was shown for intervention group x ethnicity ($p < 0.001$),
247 and for d) time visit x intervention group x ethnicity ($p < 0.01$). Similar findings were found
248 for 25(OH)D₃: b) time visit x ethnicity interaction was significant ($p < 0.0067$) and c)
249 intervention group x ethnicity interaction was significant ($p < 0.0001$) and d) a non-significant
250 trend for time visit x intervention group x ethnicity interaction ($p < 0.1$).

251

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

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

256

257 When the data for the two ethnic groups were combined, both vitamin D₂ fortification
258 products demonstrated a substantial impact upon total 25(OH)D concentrations, with a 33%
259 and 34% increase over the course of the intervention for the D2J and D2B groups
260 respectively. The vitamin D₃ fortification products demonstrated even greater effects, with the
261 D3J and D3B groups increases in total 25(OH)D in the order of 75% and 74% respectively.

262

263 When comparing across intervention groups and considering change from baseline, the D3J
264 group also demonstrated a significantly higher absolute change in total 25(OH)D
265 concentrations over the course of the intervention when compared to D2J (Δ 16.9 nmol/l [95%
266 CI 9.0, 24.8], ($p < 0.0005$), D2B (Δ 16.0 nmol/l [95% CI 8.0, 23.9], ($p < 0.0003$) and placebo (Δ
267 42.9 nmol/l [95% CI 35.0, 50.8], ($p < 0.0005$)). In addition, the D3B group demonstrated a
268 significantly higher absolute change in total 25(OH)D when compared to the D2B group (Δ

269 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],
270 ($p < 0.0005$), and the placebo group (Δ 42.3nmol/l [95% CI 34.4, 50.2], ($p < 0.0003$).

271

272 No significant difference in absolute change between the D3J and D3B groups was detected
273 over the time course of the intervention, thus indicating equivalent bioavailability (Δ
274 0.6nmol/l [95% CI -7.4, 8.6] ($p < 0.34$). Similarly, for the D2J and D2B groups, no significant
275 difference in absolute change for total 25(OH)D concentrations was detected between the two
276 groups over the course of the intervention (Δ 0.9nmol/l [95% CI -6.9, 8.7], ($p < 0.25$).

277

278 Since there were no significant interactions for ethnicity, we did not analyse further the
279 25OHD status for the Caucasian and South Asian groups separately. However we observed
280 from the data (**Table 2**) that the South Asian women appeared to have a greater response to
281 the vitamin D (both D2 and D3) compared to Caucasian women, likely due to their lower
282 25(OH)D status at baseline (< 30 nmol/l in all South Asian groups). We also observed that in
283 those South Asian women in the vitamin D₂ group, 25OHD status did not reach 50nmol/l at
284 the end of the 12 week period but those taking the vitamin D₃ juice did. When considering
285 only those South Asian participants who completed the entire intervention (n 63, 71%
286 completion), 72.7% of those South Asian women who consumed either vitamin D₃ product
287 attained levels > 50 nmol/l whereas only 55.6% of SA participants consuming either D₂
288 product met the same serum 25(OH)D threshold. For the white European women who
289 completed the study (n 228, 93% completion), all of those participants in the D3B and D3J
290 groups achieved serum 25(OH)D levels > 50 nmol/l at the end of the intervention. In contrast,
291 90.9% of participants from the D2B and 89.4% from the D2J groups met the threshold
292 of > 50 nmol/l post-intervention. When combining the D₂ groups, the attainment rate was

293 90.1%. For the placebo group, all SA women were below the 50nmol/l cut-off at the end of
294 intervention, yet 42% of EU women were maintaining total 25(OH)D levels >50nmol/l.

295

296 **Serum parathyroid hormone concentrations in two ethnic groups combined**

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

304

305 **Serum 25(OH)D₂ and 25(OH)D₃ concentrations in two ethnic groups combined**

306 Given the fact that no significant differences were detected between the juice and biscuit
307 groups within their respective vitamin D₂ and D₃ fortification strands, the groups' juice and
308 biscuit data were aggregated to explore the response of 25(OH)D₂ and 25(OH)D₃ over the
309 course of the intervention (taking into account the baseline values). As described in **Figure**
310 **2A**, for the aggregated vitamin D₂ intervention group (n 133), over the course of the
311 intervention, there was a significant increase in 25(OH)D₂ compared to both the placebo
312 (Estimated Percentage Difference [EPD] 2328.8% [95% CI 1717.4, 3113.7] ($p<0.0002$)) and
313 D₃ groups (EPD 3018.7% [95% CI 2353.3, 3864.6] ($p<0.0002$)). For the 25(OH)D₃ response
314 (**Figure 2B**), the aggregated D₃ intervention group (n 137) exhibited a significantly greater
315 response over the course of the intervention when compared to the placebo (EPD 185.8%
316 [95% CI 148.4, 228.7] ($p<0.0001$)) and D₂ groups (EPD 281.9% [95% CI 242.1, 326.3]

317 ($p < 0.0001$)), however there was also a significant difference in 25(OH)D₃ responses between
318 the D₂ 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 $2.7 \pm 2.3 \mu\text{g}$ per day (78.2% response rate, n 262). Mean intake for key nutrients
338 was as follows: Energy $7969.5 \pm 1864.6 \text{kJ}$, Total Fat $78.6 \pm 26.2 \text{g}$, Protein $72.8 \pm 18.1 \text{g}$,
339 Carbohydrate $204.7 \pm 51.7 \text{g}$ and Calcium $849.1 \pm 260.9 \text{mg}$.

340 Participants' UV exposure for the duration of the trial was minimal, with a mean exposure of
341 $0.035 \pm 0.039 \text{SED}$ pre-intervention and $0.086 \pm 0.137 \text{SED}$ post-intervention for the cohort.

342 **DISCUSSION**

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

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

367 The tests for interaction between the time visit, intervention group and ethnicity showed some
368 interesting findings: The time visit by intervention group interaction was significant across
369 the board for the primary and secondary objectives. This was the main focus of the study –
370 whether vitamin D₂ was different from vitamin D₃ with respect to changes in total 25(OH)D
371 status and their concomitant differences from the placebo group . For total 25(OH)D status,
372 where the interaction test involved ethnicity, the results were not significant, which was
373 predictable given that our results showed no difference in the absolute rise in 25(OH)D status
374 in response to fortification between white European and South Asian women. However, the
375 statistical results/trends for the ethnicity interactions with respect to 25(OH)D₂ and 25(OH)D₃
376 status are intriguing and certainly warrant further investigation.

377 Our main findings, showing greater efficacy of vitamin D₃, is supported by a meta-analysis
378 completed in 2012, which collated all studies to date that had directly compared the effects of
379 vitamin D₂ and D₃ on total 25(OH)D (14). The meta-analysis indicated that vitamin D₃ was
380 more efficacious than vitamin D₂ in raising total 25(OH)D. However the finding was mainly
381 driven by studies using large single or intermittent bolus doses of vitamin D. Studies giving
382 lower doses were largely unrepresentative, and the doses used (40-100 µg/d) were still higher
383 than (a) global public health recommendations for daily consumption, and (b) intakes
384 attainable without the use of supplements. Since the meta-analysis, there have been further
385 randomized-controlled trials comparing vitamin D₂ and D₃ at lower daily doses (25-50 µg/d),
386 although largely under-powered, that are consistent with our findings (18-20). Therefore this
387 study strengthens the current evidence base, with provision of irrefutable data from a large
388 cohort size following a robust study design.

389 An interesting result of our study is the response of the 25(OH)D metabolites; specifically the
390 response of 25(OH)D₃ to the vitamin D₂ intervention, and 25(OH)D₂ to the vitamin D₃
391 intervention. The decrease in 25(OH)D₃ that was shown in the aggregated vitamin D₂ group

392 (D2B and D2J combined; Figure 2) is consistent with previous findings from trials using daily
393 doses of 25-100 µg/d (7, 19-21). Our study also showed a decrease in 25(OH)D₂ in the
394 vitamin D₃ juice group, which has only previously been reported to have been found by
395 Binkley and colleagues, although their data were not presented as too few participants had
396 measureable 25(OH)D₂ at baseline (7). Whether this finding has not been shown in previous
397 studies due to low concentrations of 25(OH)D₂ at baseline (typically <5 nmol/L) remains
398 unclear, although Glendenning and colleagues found no change in 25(OH)D₂ in their vitamin
399 D₃ group despite having higher baseline 25(OH)D₂ (13.3 nmol/L)(12).

400 A recent study by Oliveri and colleagues (22) took a pharmacokinetic approach to understand
401 the mechanism behind the apparent difference in efficacy between vitamin D₂ and D₃. The
402 group administered a loading dose (2,500 µg) at day 0, followed by two weeks of daily
403 supplementation (120 µg/d, from day 7 to day 21) with either vitamin D₂ or D₃, and then a 56-
404 day clearance period. Their data shows that at both the post-loading dose phase (day 7) and
405 post-daily dosage phase (day 21) there is no significant difference between groups, although
406 the D₃ group had higher concentrations of 25(OH)D; yet at end of the clearance phase, the D₃
407 group had significantly higher 25(OH)D than the D₂ group. Oliveri and colleagues calculated
408 that the elimination half-life of 25(OH)D for the D₂ group was substantially shorter at 33 days
409 when compared to 82 days for the D₃ group (22).

410 It is becoming clearer from both the literature and the results of this study, that there is a
411 pronounced difference in the efficacy of vitamin D₂ and D₃ in raising total 25(OH)D. The
412 mechanisms driving this differentiating factor appear to be focussed around the effect of
413 vitamin D₂ on 25(OH)D₃, which indicates a possible mechanism encompassing competitive
414 binding and differences in binding affinity between vitamin D₂ and D₃ with the vitamin D
415 binding protein and hydroxylation enzymes. However, the shorter half-life of 25(OH)D₂
416 compared with 25(OH)D₃ (22-23) also suggests that the elimination or degradation of

417 25(OH)D is another mechanism explaining the differences in the efficacy. To further expand
418 this field and develop the mechanism, the *in vivo* behaviour of the CYP2R1 and CYP27B1
419 enzymes must be understood.

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

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 D₂ and vitamin D₃ (15
457 µg/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 D₃ is superior in raising total serum 25(OH)D
459 status when compared to vitamin D₂, 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.

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478

479 Conflict of Interest

480 LT, LW, KH, SJ, SdL, CPS, GB, SP, GC, RE, EH and JB had no conflicts of interest to
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482 Corporation Council Patent Pending) for the use of any UVB material for the prevention of
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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/m²)	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/m² – 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

Total 25(OH)D (nmol/l)	Intervention Groups				
	Placebo (<i>n</i> 65)	D2 Juice (<i>n</i> 67)	D2 Biscuit (<i>n</i> 66)	D3 Juice (<i>n</i> 70)	D3 Biscuit (<i>n</i> 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)
<i>South Asian</i>	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)
<i>White European</i>	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
<i>South Asian</i>	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)
<i>White European</i>	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
<i>South Asian</i>	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)
<i>White European</i>	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, $p \leq 0.003$; *b* – significant difference between D2B and D3B for ‘All’ participants, $p \leq 0.002$; 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)D₂, 25(OH)D₃, calcium and parathyroid hormone (PTH) concentrations at baseline, 6 weeks and 12 weeks per intervention group

	Intervention Groups				
	Placebo (<i>n</i> 65)	D2 Juice (<i>n</i> 67)	D2 Biscuit (<i>n</i> 66)	D3 Juice (<i>n</i> 70)	D3 Biscuit (<i>n</i> 67)
Week 0 (baseline)					
25(OH)D ₂ (nmol/l)	1.38 (1.05, 1.82)	0.97 (0.74, 1.27)	1.23 (0.94, 1.62)	1.14 (0.88, 1.48)	1.14 (0.87, 1.49)
25(OH)D ₃ (nmol/l)	32.1 (27.5, 37.6)	33.7 (28.9, 39.3)	33.7 (28.8, 39.4)	30.9 (26.6, 35.9)	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)	1.37 (1.10, 1.71)	28.60 (22.99, 35.57) ^{*a}	26.59 (21.30, 33.20) ^{*b}	0.82 (0.66, 1.03) ^{*a}	1.07 (0.85, 1.35) ^b
25(OH)D ₃ (nmol/l)	25.4 (22.3, 28.9) [*]	20.8 (18.3, 23.6) ^{*a}	22.7 (19.9, 25.8) ^{*b}	61.4 (54.0, 69.8) ^{*a}	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)	1.59 (1.25, 2.02)	29.54 (23.21, 37.58) ^{*a}	31.27 (24.56, 39.82) ^{*b}	0.89 (0.69, 1.14) ^{*a}	1.17 (0.91, 1.51) ^b
25(OH)D ₃ (nmol/l)	24.3 (21.2, 27.7) [*]	17.0 (14.9, 19.5) ^{*a}	21.4 (18.7, 24.5) ^{*b}	65.4 (57.1, 74.9) ^{*a}	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)D₂ and 25(OH)D₃ ($p < 0.0004$

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 $25(\text{OH})\text{D}_2$, 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 $25(\text{OH})\text{D}_3$: 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 $[(40 - \text{albumin}) \times 0.02] + \text{Calcium}$. 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)D₂. (B) 25(OH)D₃. Placebo group *n* 65, D₂ group *n* 133, D₃ 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: ▼ D₃ aggregated intervention group; ▲ D₂ aggregated intervention group; ● Placebo group

Figure 1

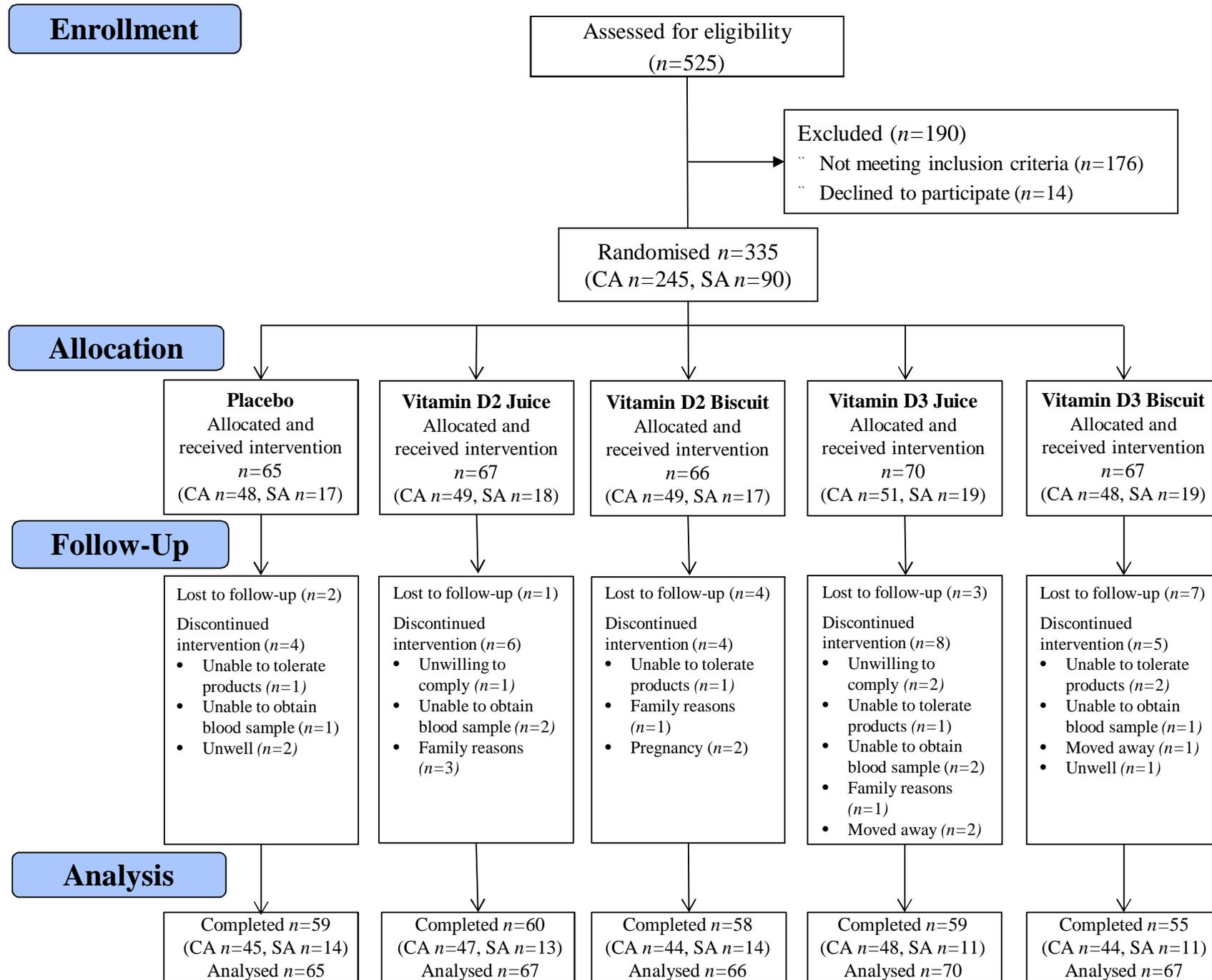


Figure 2

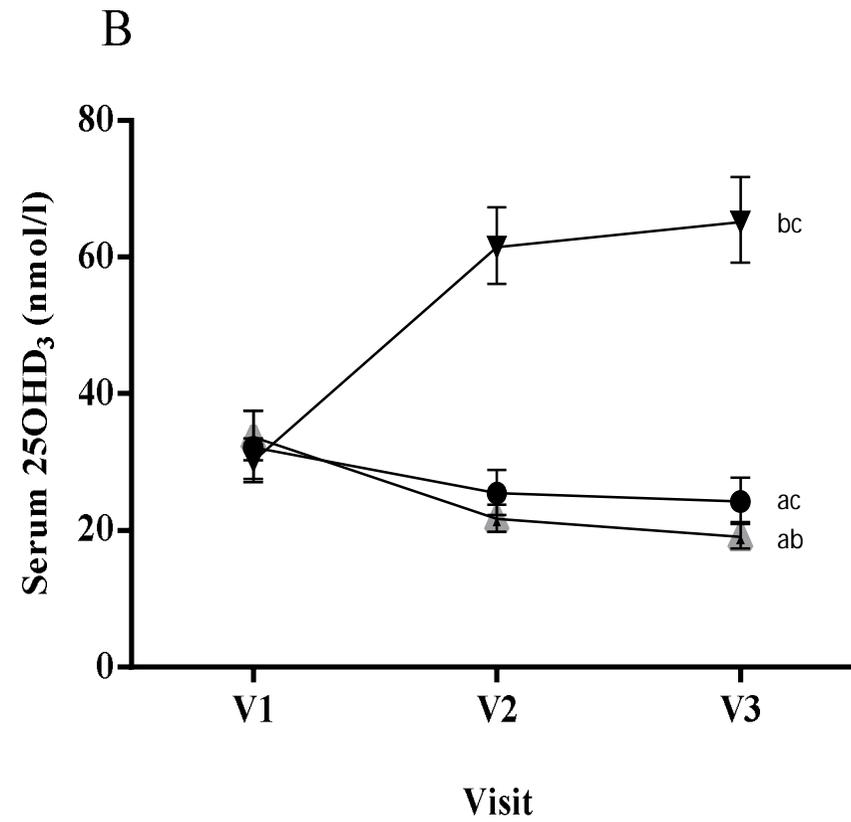
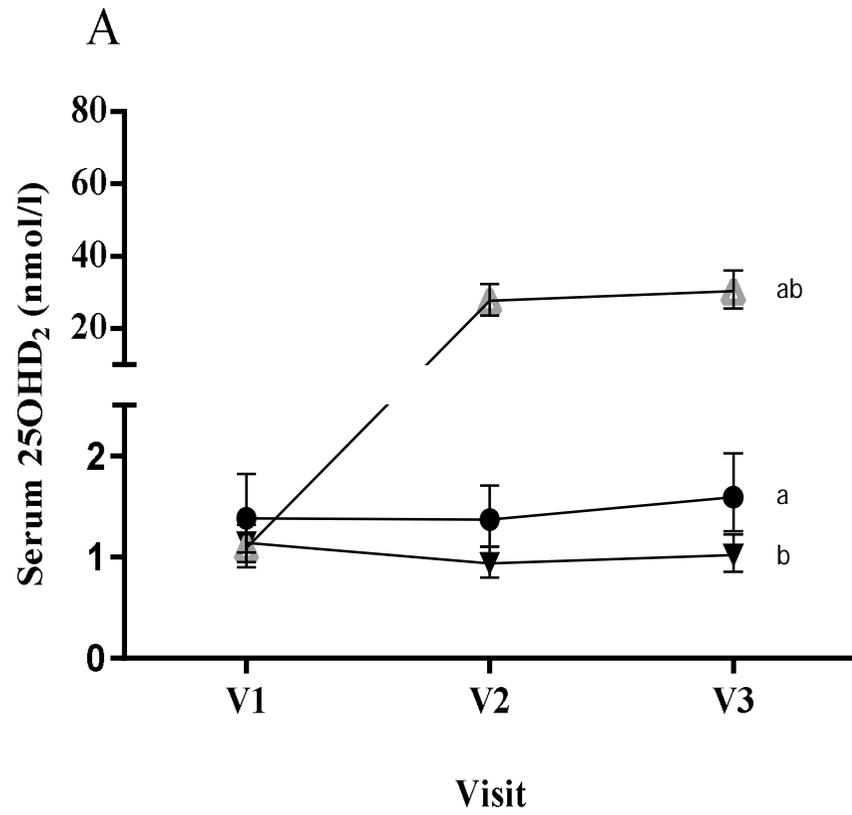


Figure 3

