Longevity of daily oral Vitamin D3 supplementation: differences in 25OHD and 24,25(OH)2D observed 2 years after cessation of a 1-year randomized controlled trial (VICtORy RECALL).

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Abstract

Purpose

To determine the longevity of vitamin D status following cessation of vitamin D3 supplementation, 2 and 3 years after a 1 year randomised double blind placebo controlled trial: (Vitamin D and Cardiovascular Risk (VICtORY)); and to investigate possible predictive factors.

Method

Of the 305 Caucasian non-smoking postmenopausal women randomised to ViCtORY (2009-2010), participants who had not taken vitamin D supplements since the trial ended were invited to attend follow up visits. Total 25-hydroxyvitamin D (25OHD) and 24,25-dihydroxyvitamin D (24,25OH2D) were measured by dual tandem mass spectrometry of serum samples following removal of protein and de-lipidation; the original RCT samples were re-analysed simultaneously. Vitamin D binding protein (VDBP) was measured by monoclonal immunoassay.

Results

In March 2012 and March 2013, 159 women (mean (SD) age 67.6 (2.1) years) re-attended, distributed between the original treatment groups: daily vitamin D3 400IU; 1000IU; and placebo. One month after the RCT ended (March 2010) the proportion of women in placebo, 400IU, and 1000IU vitamin D3 groups, respectively, with 25OHD<25 nmol/ L was 15%,0%, 0% (Chi-square p<0.001, n=46,44,54). After 2 years (March 2012) it was 22%, 4%, 4% (p=0.002, n=50,48,57); after 3 years 23%, 13%, 15% (p=0.429,n=48,45,52). The respective proportion of women with 24,25OH2D < 2.2 nmol/L were 50%, 2%, 2% (1 month, p>0.001, n=46,44,54); 42%, 33%, 12% (2y, p=0.002,n=50,48,57) and 45%, 27%, 29% (3y, p=0.138, n=47,45,51,). VDBP was a predictor of circulating 25OHD longevity (beta for VDBP in µg/ml:0.736; 95% CI 0.216-1.255,p=0.006) but not 24,25OH2D.

INTRODUCTION

Vitamin D continues to be the subject of controversy with regard to health outcomes[1,2], and the exponential increase in often inappropriate testing of 25-hydroxyvitamin D [25OHD], for assessing vitamin D status, continues to be a burden on health resources [3] . Whereas population observational studies suggest a beneficial association between vitamin D status and many health outcomes, more recent intervention studies have been less convincing [4]. A number of other structured literature reviews and meta-analyses have been performed with mixed conclusions [1,5-7]. Government evaluations of the evidence have concluded that the only in the case of musculoskeletal health is the role of vitamin D well-founded [8-10].

Lack of sunlight for cutaneous synthesis of vitamin D; and the limited amount of vitamin D present naturally in the diet; lie behind concerns about vitamin D deficiency in Northern Europe and the US. The past assumption that vitamin D made in the summer lasted over the winter months [11] has largely been dismissed on the basis of the half-life of circulating 25OHD being only a few weeks [12]. Yet the half-life estimates of 25OHD are based on extrapolation of data from short-term studies [13,14] and there are no measured long-term data on the longevity of vitamin D. The Scientific Advisory Committee of Nutrition (SACN) recommended a reference nutrient intake (RNI) of 400 IU daily vitamin D for all adults in the UK [9], compared to 600IU being recommended in the US and Europe [8,10].

'Vitamin D' intervention and dosing studies have consistently shown that the lower the starting 25OHD the greater the increase [15,16]; and progressively higher doses of vitamin D result in smaller incremental increases in 25OHD [17,18]. It is uncertain whether additional vitamin D is incompletely absorbed, or metabolised more quickly and excreted, suggesting that there is no benefit to taking higher doses; or whether it is stored (or converted to other metabolites that are either stored, or protected from degradation), allowing additional amounts to be used in the future. The role of vitamin D binding protein (VDBP) in binding 25OHD has been suggested as one way that 25OHD is

protected from degradation [19]. Most research on VDBP has focussed on adjusting 25OHD for VDBP in the belief that it is free, not total, 25OHD that is associated with health outcomes [20]. Criticisms have been raised regarding the limited data available for the equations used to estimate free 25OHD, and the monoclonal immunoassay used to measure VDBP [21]. The latter does not detect VDBP from different genotypes, which is particularly relevant when considering racial differences, as the distributions of VDBP genotypes may vary according to ethnicity [21].

Although it is only 1,25 dihydroxyvitamin D that has biological activity (circulating at concentrations of pmol/L and under homeostatic control), and 25OHD that is the accepted marker of status; there is interest in other vitamin D metabolites, particularly 24,25 dihydroxyvitamin D (24,25OH2D) with research focusing mainly on its interference in the measurement of 25OHD [22] . This metabolite, which circulates at 1/13 of the concentration of 25OHD, is the first step on the degradation pathway [23] but what role, if any, it might play in health is unknown. There are few data on the status of different vitamin D metabolites especially from intervention studies [24,25].

The VICtORy (VItamin D and CardiOvascular Risk) RCT in 2009-10 had all participants starting at the beginning of the year, when 25OHD is at its lowest in the UK, allowing direct bimonthly comparison between the treatment and placebo groups throughout the year. It found that there was no effect of daily vitamin D3 treatment (400 IU or 1000 IU) on conventional markers of cardiovascular risk at any point [26]. The aim of this RECALL study was to determine the longevity of vitamin D supplementation, by testing for differences in the proportion of women with 25OHD < 25 nmol/L, and any differences in 24,25 dihydroxyvitamin D (24,25OH2D), two and three years after the VICtORY study intervention phase had ended; and to determine possible predictive factors.

METHODS

Population and participant involvement

The women in this study were healthy non-smoking postmenopausal women mean age (SD) 64.7 (2.1) y who had taken part in a placebo controlled randomized controlled trial of vitamin D3 between January 2009 and February 2010 [26] and attended a 'decay visit' in March 2010, 1 month after study completion. The results of the VICtORy RCT, which showed no difference in markers of cardiovascular risk, inflammation, or glucose tolerance after 1 year between the 2 treatment groups (400 IU and 1000 IU daily vitamin D3) and placebo; and an identical seasonal decrease on blood pressure for the 3 groups, were presented to the volunteers (n=287 of 305 who took part) at one of three presentations. The formal presentation was followed by an informal lunch with the researchers, and the opportunity to ask questions. It meant that when the women were recalled in 2012 most had not taken vitamin D supplements since the study ended. We anticipated that one half the women originally randomised would be willing and eligible to return (n 152). Some women were taking cod liver oil capsules containing < 5 µg (200 IU) vitamin D3 a day and this was adjusted for in the statistical analysis. A group of 3 VICtORy RCT participants advised on the RECALL study design prior to its implementation, in particular their opinions on wearing UV exposure badges. Ethical approval for the RECALL study was provided by North of Scotland Research Ethics Committee (12/NS/0013), and all women gave informed consent.

RECALL visits

Women were invited to attend additional visits in March 2012 and March 2013 (two years and three years after the original trial had ended). Those who agreed to take part (n=159, mean [SD] age 67.6 [2.3] y), confirmed that they had not taken vitamin D supplements since the study ended and were not intending to take extended holidays abroad. A flow diagram showing the numbers of women at the start and end of the original RCT, and the numbers in the RECALL study is shown in Figure 1. At

both RECALL visits the women were weighed on calibrated weighing scales (Tanita Europe BV, Amsterdam, The Netherlands), their height measured by stadiometer (Holtain Ltd Crymych UK), and blood pressure measured using a Omron 705CP sphygmomanometer (Omron, Herts UK). Lean and fat body mass was obtained from whole body scans at the final RCT visit (V6) using dual energy X-ray absorptiometry (DXA; Lunar iDXA, GE Medical Systems Inc, Madison, WI) and a blood sample was taken for measurement of 25OHD. As a result of changes in analytical procedure to remove artefacts, the samples were analysed together with re-analysis of the original study samples. The researchers seeing the volunteers and analysing the data; and the laboratory carrying out the analysis remained blind to the intervention groups.

Diet had been assessed by food frequency questionnaire at 2-monthly intervals during the VICtORy trial [27]. The women again completed the FFQ at the first RECALL visit (March 2012). In March 2013, those whose diet had changed in the previous year completed an additional FFQ. Any vitamin D obtained from cod liver oil was added to the dietary vitamin D.

At each visit, skin colour was measured at the forearm, and left and right cheekbones using a hand-held chromameter (CM-2600d spectrophotometer; Konica Minolta Photo Imaging (UK) Ltd., Feltham, UK). The measurements obtained for L*(dark-light) and b*(blue yellow) axes of colour were used to estimate the individual topology angle (ITA, an indication of melanin pigment in the skin where the higher the ITA, the lighter the skin colour) according to the following equation ITA = [arctangent(L* - 50)/b*] x 180/π [28]. The women also reported their skin type [29].

In between the two RECALL visits the women were sent badges every month, to wear for 1 week to assess UV radiation exposure, which they returned by post. The UVB badges were disposable, made of polysulphone film; one was worn for 3 days and replaced by a second one on day 4 for the rest of the week. The absorbance at 330nm of the polysulphone badge was measured using a spectrophotometer (Perkin-Elmer UV/VIS Lambda 2 spectrophotometer) before and after wearing

(Δ330), and converted to standard erythemal dose (SED) using the equation: SED =10.7(ΔA330) + 14.3 $(\Delta A330)^2$ - 26.4 $(\Delta A330)^3$ + 89.1$(\Delta A330)^4$ [30]. One SED is equivalent to 100 J/m^2 UV radiation exposure. The SED estimates from each badge were summed to obtain the weekly total. Typically, a person would receive 1 SED/ day UV exposure during the summer in Aberdeen [31]. A subset of women also wore UVA badges (purchased from Scienterra Ltd, Oamaru, NZ), which were electronic devices that continuously recorded data. Algorithms (written by Maciej Gryka) were used to extract the data collected during the week they were worn by the volunteers. The badges had been calibrated with the assistance of Dr Martin Allen (Health Protection Agency, Cambridge) enabling the readings to be converted to SED equivalents. At the final visit women provided details about holidays to sunny destinations during the previous year, for testing in the statistical models. Women were also asked if they had been away for >1 month between the end of VICtORY RCT and the start of the RECALL study, and a sensitivity analysis was done excluding these women.

Laboratory Measurements

Circulating vitamin D metabolites and parathyroid hormone (PTH) were measured at the University of East Anglia. 25OHD3, 25OHD2, 24,25OH2D3 and 24,25OH2D2 were measured simultaneously by a published liquid chromatograph tandem mass spectrometry (LC-MS/MS) method [25]. In brief, 100µL of sample spiked with [2H6]-24,25-OH2D3 and [2H6]-25OHD3 as internal standards was extracted using supported liquid extraction (SLE) for removal of phospholipids followed by derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to enhance ionisation efficiency. Measurements of 25OHD3 were calibrated using commercial 25OHD3 standard materials (Chromsystems, Müchen, Germany) that were traceable to reference source NIST SRM972a. The inter-assay CV for 25OHD3 and 25OHD2 were ≤9% across the analytical range between 0-200 nmol/L, with lower limit of quantification (LLoQ) at 0.1 nmol/L. Mean assay recovery was 96 ±2%. Vitamin D external quality control (DEQAS) returns showed the accuracy bias of 25OHD3 measurements against NIST reference method was <8%. Measurements of 24,25OHD3 and 24,25OHD2 were calibrated using commercially

certified standard materials spiked into human based vitamin D-depleted serum. The inter-assay CVs were <10% across the analytical range between 0-25 nmol/L, with LLoQ at 0.1 nmol/L. Mean assay recovery was 97 ±4%. DEQAS returns on 24,25OH2D3 showed a 2% bias against all laboratory trimmed mean. PTH was measured in plasma samples using an electrochemiluminescent immunoassay (ECLIA) on a Modular Analytics E170 analyzer (Roche Diagnostics, Burgess Hill, UK). Inter/intra-assay coefficient of variation was <4% from 1 to 30 pmol/L. The assay sensitivity (replicates of the zero standard) was 0.8 pmol/L.

Vitamin D Binding Protein (VDBP)

VDBP was measured in Aberdeen using a monoclonal enzyme-linked immunosorbent assay (Human vitamin D BP QuantikineELISA Kit; R&D Systems, Oxford, UK) according to the manufacturer's instructions. The inter-/intra-assay CV was <7.4 and <6.2%, respectively.

Statistical analysis

All analyses were done using SPSS version 24 with pre-planned analysis. Dependent variables were transformed using the natural logarithm (Ln) if required. Independent t-tests were used to check the characteristics between RECALL participants and those who did not take part. Chi square tests were used to test differences in the number of women with 25OHD < 25 nmol/L and 24,25OH2D < 2.2 nmol/L between treatment groups. Linear regression was used to relate the 25OHD measurements obtained by the two different tandem mass spectrometry procedures (original and modified de-lipidation method). Repeated measures ANOVA was performed for testing differences in vitamin D metabolites and PTH according to study visit and treatment group; and to detect any differences in monthly sunlight exposure during 2012-3, between treatment groups. Mixed model analysis was undertaken to determine the predictors of 25OHD and 24,25OH2D at the RECALL visits (2-visit model). A 4-visit model compared the last four vitamin D metabolite measurements (at the end of

the VICtORy intervention, 1 month later, 2y later, and 3y later). Both models tested factors that might influence the change in vitamin D metabolites (treatment group, use of cod liver oil supplements, lean body mass, fat mass, passive smoking, dietary vitamin D and other nutrients [vitamin A, preformed retinol, calcium, protein, fat, carbohydrate and energy], sunlight behaviour, holidays abroad, skin type, VDBP). There were few data points missing for the main mixed model and the missing data were assumed to be missing completely at random.

RESULTS

Of the 265 women who completed VICtORY, 236 responded (89%): 43 (16%) taking > 400 IU daily vitamin D (some prescribed supplements as an adjunct to osteoporosis treatment) were excluded; 25 were unable (other commitments, travel, or unknown); and 9 responded positively after the deadline (Figure 1). A total of 159 women attended the RECALL visits (n=52, n=49, n=58, respectively for placebo, 400 IU, and 1000 IU- vitamin D treatment groups). There were no differences in characteristics according to their original treatment allocation except for VDBP, where mean [SD] was significantly higher for the 400IU group (296 [44]µg/ml) compared to 1000 IU (244 [89]µg/ml) (post hoc ANOVA p=0.02) (Table 1). There were no differences between the women attending and not attending the RECALL study, in terms of age, height, and starting vitamin D status, but those who took part were slightly heavier (Supplementary Table 1).

Comparison of 25OHD obtained by the original tandem mass spectrometry method with a modified method that included a de-lipidation procedure

The results of 25OHD using the original method have been reported previously[26]. The de-lipidation step introduced for the 25OHD measurements, reported here, resulted in greater recovery of 25OHD. An equation relating the two was obtained by linear regression analysis:

Ln(25OHD with delipidation)= 0.859 x Ln(25OHD with delipidation) + 0.769 (R=0.904)

The modified method gave higher mean (SD) 25OHD concentrations (n=2032 measurements for comparison: Total 25OHD: 68.6 (26.9) vs 55.4 (23.0) nmol/L; 25OHD2: 1.7 (1.1) vs 1.0 (1.4) nmol/L; 25OHD3: 66.9 (27.2) vs 54.4 (23.1) nmol/L), and fewer women with 25OHD < 25 nmol/L . The percentages of women who took part in the RECALL study with 25OHD< 25 nmol/L at the start of the RCT, using the original method was 34.6, 28.6 and 32.8 for placebo, 400 IU and 1000 IU respectively (Chi-square p=0.802); whereas using the modified method it was 13.0,8.2 and 12.7, respectively (Chi-square p=0.694).

Numbers of women below thresholds for 25OHD and 24,25OH2D

The percentage of women with 25OHD < 25 nmol/L, and 24,25OH2D < 2.2 nmol/L at each visit are shown in Figure 2. For the placebo group, the percentage with 25OHD < 25 nmol/L decreased gradually at each visit until July-August (the only visit where no-one had 25OHD < 25 nmol/L) and then gradually increased until the end of the study. For the treatment groups there was a rapid increase in 25OHD so that no participant had 25OHD< 25 nmol/L after 2 months treatment, and although mean 25OHD had decreased 1 month after the intervention ceased, no-one had fallen below the 25 nmol/L threshold (Figure 2). One month after the RCT ended (March 2010) the respective number of women in placebo, 400IU, and 1000IU vitamin D3 groups, with 25OHD<25 nmol/ L was 15%,0%, 0% (p<0.001, n=46,44,54). After 2 years (March 2012) it was 22%, 4%, 4% (p=0.002, n=50,48,57); after 3 years 23%, 14%, 17% (p=0.429, n=48,45,52). Using the 24,25OH2D marker, there was a similar pattern for the 2.2 nmol/L cut off. The respective number of women with 24,25OH2D < 2.2 nmol/L was 50%, 2%, 2% (1 month, p>0.001, n=46,44,54); 42%, 33%, 12% (2y, p=0.002, n=50,48,57) and 45%, 27%, 29% (3y, p=0.138, n=47,55,51).

The mean circulating concentrations of 25OHD and 24,25OHD (Figure 3) had similar trajectories: the placebo showing a marked season pattern with much lower circulating concentrations than either treatment group, even in summer. There was little change in 1,25OH2D and PTH (the latter two analytes were collected at selected visits only) (Figure 3).

Dietary vitamin D and sunlight

Mean (SD) daily dietary intake of vitamin D was 5.1 (3.4) μg in both 2012 and 2013 (Table 1). Cod liver oil was taken by 37 participants, averaging intake 1.2 (2.4) μg/d in these individuals and increasing overall mean (SD) dietary vitamin D intake to 6.4 (4.1) μg/d.

The sunlight exposure of the subset of women who wore badges (supplementary figure 1) showed similar monthly patterns for UVB and UVA, and no differences between treatment groups (repeated measures ANOVA). The two variables were highly correlated (Spearman coefficient 0.7, P<0.001).

Women who had been on holiday abroad for longer than one month since the study finished (n 22) had higher circulating 25OHD compared to the rest of the women (55.5[24.7] n=21 versus 46.2 [18.9] n=126), although not significant.

Mixed model analysis of 25OHD and 24,25OH2D measurements following the end of the VICtORy RCT

The mixed model which included the last four 25OHD measurements (at the end of the intervention, +1 m, +2y, and +3y) showed the time variable and the original study group to be significant predictors of 25OHD. Dietary energy intake and VDBP were additional significant predictors, with VDBP positively associated; and dietary energy negatively associated with 25OHD. Dietary vitamin D and vitamin A were not associated with 25OHD. Although dietary fat, protein, carbohydrate and dietary calcium intake appeared to be significant predictors of 25OHD, when tested independently, the relationship disappeared when dietary energy was included, due to the correlation between these macronutrients and dietary energy intake. Weight, height, age, sunlight behaviour, body fat mass, and lean mass (collected at the end of the VICtORy intervention period [27]) were not significant predictors of 25OHD (Table 2, Model 1).

The second model for the last two 25OHD measurements (2012 and 2013) tested skin colour, and participants' sunlight behaviour, including the total number of days spent abroad on holiday between the two visits. Again visit, treatment group and VDBP were significant predictors of 25OHD,

but not dietary energy intake. In this model the use of cod liver oil supplements, darker arm skin colour, and number of days spent abroad indicated higher vitamin D status. (Table 2, Model 2a). Finally a subset analysis in which there was more detailed information on sunlight exposure obtained from wearing UV detection badges, showed that overall UVB (but not UVA) exposure was a weak negative predictor of 25OHD (Table 2, Model 2b).

VDBP was not a predictor of 24,25(OH)2D (Table 2). Only 'holidays abroad' remained a positive predictor in the final 4-visit model . For the 2-visit model, as for 25OHD, supplement use was a positive predictor and there was a weak negative association with UVB and not UVA exposure.

For both vitamin D metabolites, there were significant associations with 1,25OHD (positive) and PTH (negative) when these were added; but only with 1,25OH2D in the subset model 2b. Sensitivity analysis excluding women abroad for longer than 1 month since the RCT finished showed no overall differences except for PTH no longer being a significant predictor of 25OHD in the 4-visit model.

The characteristics of women below and above the thresholds at the final RECALL visit (when there was no longer a difference between treatment groups) showed differences in 1,25(OH)2D and 25OHD before the RCT started, for 25OHD; and also supplement use for 24,25OH2D (supplementary Table 2).

DISCUSSION

We believe that this is the only long-term study to examine vitamin D status after completion of a placebo-controlled RCT of low dose, daily vitamin D3. Our data, showing that vitamin D supplementation is still evident 2-years but not 3-years after cessation, are consistent with a 1-year single-arm study in 45 nursing home residents providing 5000 IU vitamin D daily from vitamin D-fortified bread, which showed that 25OHD measured in 23 residents was higher compared to baseline 1 year after the study (64.9 ± 24.8 nmol/L) but not 3 years (28.0 ± 15.0 nmol/L) [32]. They support our longitudinal data over 3 years, indicating that for the majority, circulating 25OHD does not fall below a set minimum[33].

We hypothesise that during winter at high latitude, circulating 25OHD is prevented from falling below a set point for a period, either being topped up from stores when required, or being protected from degradation to some extent. It is only when the store or protected 25OHD has been exhausted that people become at risk of vitamin D deficiency: when usage begins to outstrip provision, and the conversion to 1,25OH2D becomes less efficient.

Although vitamin D stored in the body was considered important in maintaining 25OHD over winter[34], other evidence from pig models [35] suggest that the size of vitamin D stores is limited [36]. There is some uncertainty regarding the exact contribution that sunlight and diet provide to the overall vitamin D economy [37,38], although direct observations show that 80%-90% 25OHD comes from summer sunlight in the UK, with 25OHD increasing rapidly in summer, against a background where dietary vitamin D remains constant [39]. A few minutes exposure to summer UVB is required to make sufficient vitamin D [40] but it is unclear how much is stored long-term, compared to vitamin D from the diet. It is possible there may be sunlight/ oral vitamin D interactions, as VICtORy treatment doses were not additive, contributing less to total 25OHD in the summer, compared to autumn [18].

VDBP was a factor affecting the half-lives of 25OHD2 and 25OHD3 [19]. Our data suggest that VDBP may play a minor role in extending the half-life of circulating 25OHD in this population, but it is likely there must be other mechanisms which preserve 25OHD. VDBP was measured at one visit only (March 2010, the same month as the two RECALL visits). The downside of the monoclonal immunoassay in failing to detect other genotypes of VDBP [21] may be less relevant within a single Caucasian population. It is possible that VDBP concentrations could change according to season, and affect how much 25OHD is metabolised, but we cannot test that hypothesis in this study. Jamil *et al*. found that VDBP was lower in spring compared to autumn in South East Asians living in Aberdeen, UK [41].

Other predictors were similar to those tested previously [31]. We did not find that individual dietary vitamin D intake predicted vitamin D status, although use of cod liver oil capsules (providing an additional 200 IU vitamin D) was important. The finding that darker skin colour, and holidays abroad between the recall visits (2012-2013) were associated with greater 25OHD is in keeping with more sunlight exposure leading to greater cutaneous synthesis of vitamin D. The weak negative association between UVB exposure obtained from the badges and 25OHD was unexpected, but may indicate some interaction between diet and sunlight as suggested earlier; or reflect that once sufficient vitamin D is synthesised, spending longer outside will not result in more vitamin D [42]. The benefit of holidays abroad and cod liver oil supplements has been noted in our cross-sectional and longitudinal studies [43] [44]. Three years after the RCT, 17% of participants had 25OHD < 25 nmol/L, with the main difference between those below and above the cut-off being the 25OHD measurement before the intervention started. Whether women revert to original behaviour after taking supplements (eg sun avoidance, diet low in vitamin D) or there is genetic predisposition to lower 25OHD[45] is unknown. Whatever the reason, our data show that small amounts of daily vitamin D are effective in preventing deficiency in this group. Tracking of 25OHD over 10 years has been noted in longitudinal studies [31,46].

Comparing the metabolite 24,25(OH)2D with the established marker of status, 25OHD, showed similar trajectories in response to supplementation, with perhaps a greater difference in 24,25(OH)2D response between 400IU and 1000IU vitamin D3 than for 25OHD, indicating a lower 25OHD:24,25OH2D ratio. This is consistent with observations that 25OHD:24,25OH2D ratios are higher when 25OHD is < 50nmol/L [25]. There were more women below the 24,25OH2D cut-off of 2.2 nmol/L compared to the 25OHD cut-off of 25 nmol/L. Unlike 25OHD, VDBP had no association with 24,25OHD longevity. Although we tested 1,25(OH)2D and PTH as additional variables in the models, the dependency is likely to be reversed, with 25OHD and 2425OH2D affecting 1,25(OH)2D positively and PTH negatively.

The accepted 25OHD half-life of a few weeks has been based on extrapolation of short-term data [13]. If there are feed-back mechanisms that prevent the continued rate of decay beyond a set minimum, these would be missed. If we had extrapolated 25OHD from our 1-month decay visit, which showed a rapid initial decrease after treatment ceased, we would have concluded that any difference in 25OHD would have disappeared within months. Our measured data at two years show that was not the case. Our findings indicate that it will be those who year-on-year do not get enough sunlight and who do not reach the RNI who are going to suffer frank vitamin D deficiency, leading to symptoms; as any protective mechanisms cannot keep going indefinitely.

SACN's risk assessment concluded that, as it could not recommend how much summer sunlight exposure was required to maintain adequate vitamin D status in the winter, the RNI of 400 IU should apply to all adults [9]. It was not within SACN's remit to consider approaches to achieve this. It remains a concern that those who are deemed most at risk of vitamin D deficiency do not get enough vitamin D. Despite the RNI for vulnerable groups existing since 1991, with recommendation of vitamin D supplements, it is clear that this message has not been reaching many in the 'at risk' groups. It is likely that any voluntary fortification of foods by manufacturers would necessitate surcharges that might render such items unaffordable to those most in need; and that mandatory fortification of

foods most likely to be consumed by different populations should be considered. Our data imply that the amounts needed are small enough not to place any risk to those currently getting sufficient vitamin D through a combination of summer sunlight and diet.

We did not find any influence of other nutrients, for example calcium considered to be 'vitamin D sparing' [47], or vitamin A; but the FFQ may be insufficiently sensitive to detect small differences. The interaction of other nutrients with vitamin D may be more important in predicting health outcomes. Other limitations of the RECALL study are that the women who took part could be different from those randomised to the RCT, but we found no significant difference in 25OHD, nor the percentage below the deficiency cut-off. Although it is possible that some women, despite their stating otherwise, had taken vitamin D supplements since the study finished; it is unlikely that this applies to everyone, as they had been presented with results showing that there was no benefit of vitamin D supplementation on cardiovascular risk markers. If those on placebo had started taking vitamin D, this would have narrowed the margins between the treatment groups. In either scenario, the women would have had to purchase vitamin D supplements themselves to continue. The generalisability of the study is restricted to outwardly healthy Caucasians in the UK, and highlights the need for concentrating resources on ethnicities at increased risk of vitamin D deficiency.

The strengths are that the study was carried out at 57°N, with no UVB radiation between April and October, and averaging less summer sunlight exposure than the rest of the UK[39]. The daily doses of 400 IU and 1000 IU vitamin D3 are relevant to population recommendations, the former being the current UK RNI (400IU), and well below recommended safe upper limits. All volunteers started the original study in January-March, and the decay and RECALL visits were in the same month (March). In addition, the improved methodology for measurement of vitamin D metabolites, with all samples being analysed together, ensures robustness of the measurements.

In conclusion, 400 IU (or 1000 IU) vitamin D3 taken daily for 1 year still showed benefits over placebo 2 years after the supplementation stopped, in terms of reducing the number of people with 25OHD

< 25 nmol/L. The benefit was no longer significant after 3 years. The findings support the RNI of 400 IU daily vitamin D in adults in order to protect the UK population from risk of vitamin D deficiency; and appropriate risk management strategies should be implemented. It is recommended that 25OHD measurement by tandem mass spectrometry adopts a de-lipidation procedure to allow better comparisons between studies and stored samples.

Competing Interests

All authors have completed the ICMJE uniform disclosure form at www.icmje.org/coi_disclosure.pdf. JCYT, ADW, LSA and WDF declare no support from any organisation for the submitted work, no financial relationships with any organisations that might have an interest in the submitted work in the previous 3 years; no other relationships or activities that could appear to have influenced the submitted work. HMM and AG acknowledge part-support from UK Food Standards Agency, and the Department of Health for the submitted work, but no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements

This work was funded partly by the UK Food Standards Agency, and the Department of Health . Any views expressed are the authors own. Original trial registration: Vitamin D effects on cardiovascular disease risk (VICtORy) study at controlled‐trials.com as ISRCTN20328039 (http://controlled‐trials.com/ISRCTN20328039/).

We wish to thank all the women volunteers taking part in this study; Karen Secombes, Minimol Paulose and Jenny Scott for assistance with venesection; Martin Allen, Health Protection Agency, Cambridge for help in standardising the UVA badges; Maciej Gryka for creating the algorithms used to extract the data from the UVA badges; and Hamish Macdonald for assistance in generating the figures.

Authors roles: HMM (principal investigator) had full access to all of the data in the study and takes responsibility for its integrity and the accuracy of the data analysis. She was involved in the study design, obtaining funding, study management, and the writing of the manuscript. AG and AW were responsible for the day-today running and cleaning of data for the VICtORy RECALL study, and the original VICtORy RCT, respectively. LSA provided statistical advice and interpretation. JT and WDF analysed and interpreted the measurements for vitamin D metabolites and PTH. All authors critically appraised the manuscript.

Role of the sponsor: The sponsor, Research and Innovation, University of Aberdeen, UK, was responsible for confirming proper arrangements to initiate, manage, monitor, and finance this RCT as designated by the Scottish Executive Health Department Research Governance Framework for Health and Community Care and the Department of Health Research Governance Framework for Health and Social Care 2nd Edition (2006)‑Confirmation of the Role of Sponsor.

Table 1 Characteristics of RECALL participants (measured in 2012 unless otherwise indicated) according to treatment allocation for the original VICtORy RCT

Mean (SD)	Placebo	400 IU vitamin D3	1000 IU vitamin D3	P
n	52	49	58	
Age (y)	67.5 (2.1)	67.4 (1.8)	68.0 (2.2)	0.222
Weight (kg)	69.6 (11.3)	70.0 (12.7)	71.1 (11.1)	0.771
Lean mass (kg)	38.6 (3.7)	38.3 (4.6)	38.5 (4.4)	0.804
Fat mass (kg)	29.0 (8.0)	29.1 (8.8)	30.0 (8.4)	0.806
Systolic Blood Pressure (mm Hg)	128 (16)	129 (16)	127 (15)	0.842
Diastolic Blood Pressure (mm Hg)	75 (8)	75 (9)	76 (7)	0.926
n	50	47	54	
vitamin D (diet only)(µg)	5.5 (3.6)	5.2 (2.9)	4.8 (3.5)	0.517
vitamin D (diet + supplements) (µg)	6.3 (4.4)	6.1 (3.4)	6.7 (4.4)	0.786
Dietary calcium (mg)	1327 (441)	1242 (741)	1269 (630)	0.784
VDBP (µg/L)	263 (90)	296 (97)	244 (89)	0.021
PTH (pmol/L)	5.6 (1.7)	5.2 (2.1)	5.0 (1.3)	0.223
n	52	49	58	
25OHD (nmol/L) at RCT start (in 2009)	36.1 (18.7)	33.3 (12.6)	33.2 (15.5)	0.559
25OHD (nmol/L) at end of	32.6 (15.6)	66.6 (20.6)	74.6 (20.1)	<0.001

RCT (in 2010)				
1,25OH2D (pmol/L) at start of RCT*	135 (42)	138 (38)	132 (42)	0.700
1,25OH2D (pmol/L) at end of RCT*	127 (42)	145 (46)	134 (42)	0.098
n	46	49	55	
25OHD (nmol/L) at RCT start (after de-lipidation)	43.6 (21.1)	46.2 (19.2)	43.7 (21.7)	0.789
25OHD (nmol/L) at end of RCT (after de-lipididation)	43.6 (18.9)	84.8 (17.2)	91.1 (24.3)	<0.001
24,25OHD (nmol/L) at RCT start (after de-lipidation)	2.9 (1.9)	3.1 (1.5)	3.0 (2.0)	0.893
24,25OHD at end of RCT (after de-lipidation)	2.8 (1.5)	6.4 (1.6)	7.7 (2.7)	<0.001
Categories				
Time abroad > 1 month since RCT	14.3	20.8	9.3	0.254
Passive smoker (%)	7.8	4.2	12.1	0.336

* 1,25OH2D measured by immunoassay

Supplementary Table 1. Differences in characteristics, measured at randomization for the VICtORY RCT (in 2009), between RECALL participants and those who did return for the RECALL visits (in 2012)

Mean (SD)	RECALL	VICtORy non-returners	p
n	159	146	
Age (y)	64.7 (2.1)	64.5 (2.3)	0.499
Height (cm)	161 (6)	161 (6)	0.425
Weight (kg)	70.6 (11.1)	67.4 (12.8)	0.018
Grip strength	22.3 (4.8)	22.5 (4.9)	0.784
25OHD (nmol/L) *	34.3 (15.8)	33.4 (13.6)	0.915
1,25OH2D (pmol/L)**	139 (46)	137 (41)	0.714
n	158	137	
Dietary vitamin D (µg/d)	5.0 (2.7)	5.7 (4.8)	0.119
Dietary calcium (mg)	1272 (528)	1285 (482)	0.822
n	153	140	
Sunlight exposure baseline (SED/ week)	0.85 (0.92)	0.89 (1.18)	0.761
n	151	114	
Sunlight exposure july-august (SED/ week)	6.15 (5.02)	7.13 (10.03)	0.302

* 25OHD measured by the tandem mass spectrometry but no de-lipidation of samples

**1,25OH2D measured by immunoassay

SED standard erythemal dose (100 J m-2) estimated from polysulphone badges

Table 2 Mixed model analysis to determine predictors of (A) 25OHD and (B) 24,25(OH)2D following cessation of supplements.

	(A) 25OHD (nmol/L)			*(B) 24,25OH2D (nmol/L)*		
Independent Variables	Beta	95% CI	P	Beta		P
Model 1 (4-visit) (n 158)						
Constant	3.919	3.673-4.164	<0.001	1.264	1.112 to 1.415	<0.001
Visit						
Study end	0.493	0.349 to 0.636	<0.001	0.566	0.448 to 0.684	<0.001
+1 month	0.376	0.238 to 0.513	<0.001	0.476	0.368 to 0.585	<0.001
+2 year	0.123	0.036 to 0.210	0.006	0.132	0.046 to 0.217	0.003
+3 year	0					
Treatment group						
placebo	-0.745	-0.869 to -0.620	<0.001	-0.951	-1.090 to -0.811	<0.001
400 IU	-0.080	-0.208 to 0.048	0.216	-0.165	-0.306 to -0.024	0.022
1000 IU	0					
VDBP (µg/L)	0.736	0.216 to 1.255	0.006			
Dietary energy intake (J/d)	-0.030	-0.048 to -0.011	0.002			

Holiday abroad yes no				0.170	0.067 to 0.274	0.001
UVB year (SED)	0.479×10^{-3}	0.044×10^{-3} to 0.914×10^{-3}	0.031			
Additional variables						
1,25OH2D (pmol/L)	2.334×10^{-3}	1.266×10^{-3} to 3.403×10^{-3}	<0.001	2.997×10^{-3}	1.775×10^{-3} to 4.218×10^{-3}	<0.001
PTH (pmol/L)	-0.039	-0.073 to -0.005	0.026	-0.068	-0.104 to -0.032	<0.001
Model 2a (2-visit) (n 151)						
Constant	4.158	3.862 to 4.634	<0.001	1.338	1.126 to 1.545	<0.001
Visit (+2 year vs +3 year)	0.082	0.020 to 0.144	0.010	0.133	0.048 to 0.218	0.002
Treatment group						
placebo	-0.203	-0.357 to -0.050	0.010	-0.273	-0.480 to -0.067	0.010
400 IU	-0.112	-0.273 to 0.049	0.172	-0.162	-0.372 to +0.049	0.132
1000 IU	0					
Supplements	+0.167	-0.321 to -0.013	0.034	0.356	0.151 to 0.561	0.001
VDBP ug/L	0.645	-0.002 to 1.292	0.051			
Holidays (days abroad)	+0.0038	0.0005 to 0.0071	0.025	0.0053	0.0008 to 0.0097	0.020

Arm skin colour (ITA)	-0.0087	-0.0166 to -0.0009	0.029			
Additional variables						
1,25OH2D (pmol/L)	3.179 x 10^{-3} *	1.740 x 10^{-3} to 4.618 x 10^{-3}	<0.001	4.203 x 10^{-3}	2.271x10^{-3} to 6.135 x10^{-3}	<0.001
PTH (pmol/L)	-0.045 *	-0.076 to -0.129	0.006	-0.058	-0.100 to -0.015	0.008
*VDBP, Supplements and Skin colour no longer significant predictors						
Model 2b (2-visit, sub-set) (n 73)						
Constant	4.168	3.897 to 4.456	<0.001	1.713	1.296 to 2.130	<0.001
Visit (+2 year vs +3 year)	0.133	0.053 to 0.213	0.001	0.176	0.060 to 0.292	0.003
Treatment group						
placebo	-0.176	-0.381 to 0.029	0.092	-0.319	-0.621 to -0.018	0.038
400 IU	-0.034	-0.247 to 0.179	0.751	-0.155	-0.468 to 0.157	0.326
1000 IU	0					
Supplements	0.420	0.203 to 0.636	<0.001	0.560	0.243 to 0.877	0.001
UVB-April-March	-0.001	-0.002 to +0.000	0.077 *	-0.0016	-0.0032 to 0.0001	0.061 *
Additional variable						
1,25OH2D (pmol/L)	3.800 x10^{-3}	1.952 x10^{-3} to 5.648 x10^{-3}	<0.001	3.698 x10^{-3}	0.946 x10^{-3} to 6.450 x10^{-3}	0.009
	* P=0.030 when additional variable added			* P=0.036 when additional variable added		

Model 1. Repeated measures was based on final VICtORy visit, +1m, +2y, and +3y time-points and included the main outcome measures 25OHD and 24,25OH2D (log transformed). Repeated variables tested included weight, dietary vitamin D and other dietary nutrients, 1,25(OH)2D, and PTH. Fixed variables tested included: visit, original treatment group, supplement use (CLO), total days holiday, VDBP, SED from VICtORy, sunlight behaviour

Model 2a. Repeated measures was based on +2y and +3y time points only, and included the main outcome measurement. Repeated variables tested included weight, dietary vitamin D and other dietary nutrients, skin colour, 1,25(OH)2D, and PTH. Fixed variables tested included original treatment group, age, total days holiday, sunlight behaviour, VDBP

Model 2b. As for model 2a above but for the subset of women who undertook sunlight exposure measurements. Exposure to UVA and UVB were tested separately

Supplementary Table 2 Characteristics of between women below and above thresholds for (A) 25OHD (25 nmol/L) and (B) 24,25OH2D (2.2 nmol/L) at the final RECALL visit

	(A) 25OHD: 25 nmol/L			*(C) 24,25OHD: 2.2 nmol/L*		
Mean (SD)	Below threshold n=25	Above threshold n=120	P	Below threshold n=48	Above threshold n=95	P
Age (y)	65.0 (2.3)	64.5 (2.0)	NS	64.8 (2.2)	64.5 (1.9)	NS
Weight (kg)	71.9 (18.4)	70.6 (10.7)	NS	71.0 (12.8)	70.2 (10.5)	NS
VDBP (ug/ml)	250 (91)	269 (93)	NS	265 (91)	266 (95)	NS
Energy intake (MJ/d)	9.18 (2.88)	8.97 (3.24)	NS	9.44 (4.21)	8.81 (2.56)	NS
Dietary fat (g/d)	82.5 (34.4)	81.8 (32.3)	NS	86.4 (40.5)	80.8 (29.8)	NS
Dietary protein (g/d)	92.3 (28.6)	92.1 (35.30	NS	95.0 (41.5)	90.9 (30.5)	NS
Dietary vitamin D (ug/d)	5.5 (3.1)	5.1 (3.4)	NS	5.57 (3.5)	5.0 (3.3)	NS
Dietary calcium (mg/d)	1330 (608)	1247 (491)	NS	1299 (665)	1243 (420)	NS
UVB June (SED)	17.4	17.3	NS	17.9 (14.8)	17.3 10.6)	NS
UVB July (SED)	23.0	20.4	NS	23.4 (19.5)	19.7 (14.5)	NS
UVB Aug (SED)	24.8	24.2	NS	24.2 (15.6)	24.2 (14.0)	NS
Holidays win12-spr13	11 (17)	19 (21)		15 (19)	20 (22)	

25OHD start V0	33.9 (15.7)	45.5 (20.0)	<0.01	35.4 (15.9)	47.9 (20.1)	<0.01
25OHD summer V3	72.2 (28.6)	82.1 (20.5)	NS	75.5 (24.8)	82.8 (19.9)	0.07
25OHD end V6	67.5 (38.1)	76.0 (27.1)	NS	66.8 (33.5)	78.3 (25.4)	0.04
Arm skin colour (ITA)	55.6 (6.1)	52.5 (5.4)	0.01	53.5 (5.8)	52.7 (5.4)	NS
1,25OH2D pmol/L V9	77.3 (35.2)	96.2 (35.2)	0.02	82.3 (29.4)	99.1 (37.2)	0.08
PTH pmol/L V9	5.5 (2.0)	5.3 (1.6)	NS	5.7 (1.9)	5.2 (1.5)	NS
Categories						
% in placebo, 400 IU and 1000 IU	44, 24, 32	31, 32, 37	NS	44, 25, 31	27, 35, 38	NS
Supplement user %	12.0	25.0	NS	12.5	28.4	0.03
Passive smoker	12.5	7.6	NS	6.4	9.5	NS

Figure 1 Participant flow diagram

Figure 2 Deficiency thresholds (A)25OHD (B) 24,25OH2D

Figure 3 Mean (A) 25OHD (new method) (B) 24,25OH2D (C) 1,25OH2D (D) PTH

Supplementary Figure 1 Monthly UV exposure throughout 2012-2013 in a subset of VICtORY RECALL

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