

Effect of vitamin D supplementation on selected inflammatory biomarkers in older adults: a secondary analysis of data from a randomised, placebo-controlled trial

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Abstract

Observational studies have suggested that 25-hydroxyvitamin D (25(OH)D) levels are associated with inflammatory markers. Most trials reporting significant associations between vitamin D intake and inflammatory markers used specific patient groups. Thus, we aimed to determine the effect of supplementary vitamin D using secondary data from a population-based, randomised, placebo-controlled, double-blind trial (Pilot D-Health trial 2010/0423). Participants were 60- to 84-year-old residents of one of the four eastern states of Australia. They were randomly selected from the electoral roll and were randomised to one of three trial arms: placebo (n 214), 750 μ g (n 215) or 1500 μ g (n 215) vitamin D₃, each taken once per month for 12 months. Post-intervention blood samples for the analysis of C-reactive protein (CRP), IL-6, IL-10, leptin and adiponectin levels were available for 613 participants. Associations between intervention group and biomarker levels were evaluated using quantile regression. There were no statistically significant differences in distributions of CRP, leptin, adiponectin, leptin:adiponectin ratio or IL-10 levels between the placebo group and either supplemented group. The 75th percentile IL-6 level was 2.8 pg/ml higher (95% CI 0.4, 5.8 pg/ml) in the 1500 μ g group than in the placebo group (75th percentiles: 11.0 *v.* 8.2 pg/ml), with a somewhat smaller, non-significant difference in 75th percentiles between the 750 μ g and placebo groups. Despite large differences in serum 25(OH)D levels between the three groups after 12 months of supplementation, we found little evidence of an effect of vitamin D supplementation on cytokine or adipokine levels, with the possible exception of IL-6.

Key words: Vitamin D: Supplementation: Cytokines: Adipokines

Chronic inflammation underlies many pathological processes including several types of cancer⁽¹⁾, CVD⁽²⁾ and autoimmune disease⁽³⁾. Animal and *in vitro* studies have identified a number of mechanisms by which the most biologically active form of vitamin D, 1,25-dihydroxyvitamin D (1,25(OH)₂D), may reduce inflammation⁽⁴⁾. For example, treatment with 1,25(OH)₂D suppresses the NF- κ B pathway⁽⁵⁾, thereby reducing downstream expression of pro-inflammatory cytokines. By acting as a ligand-activated transcription factor, the vitamin D receptor

(VDR) can also directly regulate cytokine gene transcription⁽⁶⁾. In addition, the VDR is expressed in adipocytes that are responsible for the production of the adipokines adiponectin and leptin. These have anti- and pro-inflammatory properties, respectively, but investigations of the influence of 1,25(OH)₂D on their secretion are scarce^(7,8).

Serum concentrations of the intermediate product 25-hydroxyvitamin D (25(OH)D) are used to measure a person's vitamin D status. Many observational studies suggest

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; CRP, C-reactive protein; LAR, leptin:adiponectin ratio; RCT, randomised controlled trial; QR, quantile regression.

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that 25(OH)D concentration is inversely associated with pro-inflammatory markers and positively associated with anti-inflammatory markers^(9–14). For example, studies have reported an inverse relationship between serum 25(OH)D levels and circulating concentrations of C-reactive protein (CRP)^(9,10), IL-6⁽⁹⁾ and leptin⁽¹¹⁾, and a positive association with adiponectin levels^(12–14). Other studies suggest a less straightforward relationship. Both pro- and anti-inflammatory cytokines levels were found to be down-regulated in summer in association with increased serum 25(OH)D levels⁽¹⁵⁾. In contrast, an observational analysis of data from a trial suggested that supplementation with vitamin D increased the levels of both pro- and anti-inflammatory cytokines in people with initially low serum 25(OH)D levels⁽¹⁶⁾.

In some trials, supplementation with vitamin D has been associated with significant reductions in circulating concentrations of CRP^(17,18) and IL-6^(19,20), and significant increases in IL-10⁽²¹⁾ and adiponectin⁽²²⁾. However, most trials with significant results were carried out in patients with a specific disease condition rather than in 'healthy' participants. Results from trials that used people without apparent disease were largely null, although these used relatively small sample sizes (n 23–307)^(23–29). A pooled analysis of data from four trials, none of which was population-based, found no significant change in CRP following high-dose supplementation with vitamin D⁽³⁰⁾.

Many people without a specific disease may still have chronic, low-grade inflammation, especially if exposed to toxic chemicals (e.g. tobacco smoke), or if overweight or obese^(31,32). Given the possible consequences for the prevention of cancer and CVD, it is worth investigating whether vitamin D supplementation can dampen subclinical inflammation in apparently healthy people. We used secondary data from our randomised controlled trial (RCT) of vitamin D supplementation in a population-based sample of older Australians, which demonstrated large effects of supplementation on serum 25(OH)D⁽³³⁾, to assess the effect of supplementary vitamin D on circulating inflammatory markers (CRP, leptin, adiponectin, leptin:adiponectin ratio (LAR), IL-6 and IL-10). To the best of our knowledge, this is one of the largest and, hence, most highly powered studies of the possible effect of vitamin D on inflammation, with relevance extending beyond highly specific patient groups.

Methods

Study population and design

The methods and protocols of the study have been described⁽³³⁾. Briefly, the Pilot D-Health trial 2010/0423 was a population-based, randomised, placebo-controlled, double-blind chemoprevention trial of vitamin D₃ in older adults. This study was conducted according to the guidelines laid down in the 1964 Declaration of Helsinki, and all procedures involving human subjects were approved by The Human Research Ethics Committee at the QIMR Berghofer Medical Research Institute. Written informed consent was obtained from all participants. Participants consented to blood samples being stored indefinitely and for them to be used in studies related to vitamin D. The QIMR Berghofer Medical Research Institute Human Research Ethics Committee approved the use of samples for this study.

We recruited 644 people aged 60–84 years who were residents of one of the four eastern states of Australia, using the Australian Electoral Roll as the sampling frame. Participants were sampled in strata of 5-year age bands, sex, state and location of residence (capital city and elsewhere). We excluded anybody who was taking ≥ 10 μg of vitamin D/d or who had a history of kidney stones, hyperparathyroidism, osteomalacia, osteoporosis or sarcoidosis. Recruitment took place between October 2010 and March 2011, and 10% of people invited were randomised. Participants were randomly allocated to one of three trial arms: placebo (n 214), 750 μg (n 215), or 1500 μg (n 215) vitamin D₃ (cholecalciferol), each taken once per month for 12 months. Participants, study investigators and staff were unaware of the treatment allocation until analysis of the primary outcome (change in serum 25(OH)D level) was complete. Approximately 95% of participants completed the study and 96% reported taking at least ten of their twelve study tablets.

Blood samples and laboratory analyses

Non-fasting blood samples were collected in serum separator tubes at study entry and after the intervention period and transported cold by overnight courier to the Queensland University of Technology (Brisbane, Australia). Participants were requested to provide a post-intervention blood sample within 2 weeks of the last tablet taken. Samples were centrifuged at 4°C for 15 min at 2000 **g**, and the serum was stored at –80°C.

Pre- and post-intervention serum 25(OH)D concentrations were measured in a single batch using a commercial chemiluminescent immunoassay (LIAISON[®] 25(OH)D Vitamin D TOTAL Assay; DiaSorin, Inc.). Intra- and inter-assay variabilities were 3–6 and 6–9%, respectively. The laboratory undertaking the testing was certified by the international Vitamin D External Quality Assessment Scheme.

We measured non-fasting serum levels of five inflammatory markers in post-intervention blood samples: IL-6, IL-10, leptin, adiponectin and CRP. Levels of IL-6, IL-10, leptin and adiponectin were measured using commercial sandwich ELISA kits (Merck Millipore) using a Multiskan Primary EIA V. 2.3 device with Labsystems Genesis V3.03 software, and levels of CRP were measured using the high-sensitivity CardioPhase hsCRP assay on the BN[™] II system (Siemens Healthcare Diagnostics). The inter- and intra-assay CV were <10 and <15% for all biomarkers. The minimum detectable concentrations for IL-6, IL-10, leptin, adiponectin and CRP were 1.6 pg/ml, 2 pg/ml, 0.78 ng/ml, 0.1 μg /ml and 0.175 mg/l, respectively. All assays for inflammatory markers were performed by Sullivan Nicolaides Pathology (Indooroopilly, Australia) according to the manufacturers' instructions.

Questionnaires

Within 2 weeks of having blood drawn at study entry and exit, participants were asked to complete questionnaires in which they reported information about their height, weight, skin phenotype, smoking and alcohol consumption, history of cancer, diabetes or CVD, physical activity, time outdoors, overall health and dietary intake of vitamin D.

Statistical analysis

We excluded participants for whom no blood sample was available for the analysis of inflammatory markers. Cytokine levels below the minimum detectable concentration were assigned a value equal to the minimum detectable concentration for that biomarker. Adipokine levels outside the appropriate range of the assay were assigned the lower or upper bound as appropriate. The LAR⁽³⁴⁾ was calculated by dividing the leptin level (ng/ml) by the adiponectin level (µg/ml).

Comparisons of post-intervention levels of serum 25(OH)D were made between intervention groups using two-sample *t* tests. As biomarker distributions were right-skewed, we present their descriptive statistics as median (interquartile range (IQR)).

We used quantile regression (QR) to evaluate the association between intervention group and each biomarker level. QR is used to model a specified percentile of a response variable, conditional on one or more predictors. It is therefore similar to linear regression, which models the conditional mean of the response variable. The distribution of some biomarkers remained right-skewed following a log transformation. QR makes no assumptions regarding the distribution of the regression residuals and therefore offered an advantage over linear regression. Further, QR was preferable to Wilcoxon's rank sum test because it permitted the inclusion of interaction terms.

We compared median levels of CRP, leptin, adiponectin and LAR, and 75th percentile levels of IL-6 and IL-10. These were meaningful comparisons given the shapes of biomarker distributions. In particular, as IL-6 and IL-10 levels were very right-skewed, we were interested in whether vitamin D supplementation might have a dampening effect on more 'extreme' levels of these biomarkers.

Intervention group was treated as a categorical predictor in QR models, with the placebo group used as the reference level. For CRP, leptin, adiponectin and LAR, QR coefficients represent the estimated difference in medians between an actively supplemented group (either the 750 or 1500 µg group) and the placebo group. For IL-6 and IL-10, the coefficients represent the estimated difference in 75th percentiles. 95% confidence intervals for each coefficient are interpreted in the usual way; a CI that does not contain 0 indicates that the percentile differs significantly between the placebo and supplemented groups. Wald's statistic was used to assess the overall significance of the intervention group. To test for linear trends, we fitted models with intervention group treated as a continuous variable, with the values 0, 1 and 2 corresponding to the placebo, 750 and 1500 µg groups, respectively.

To test whether the effect of supplementation was modified by sex, age (<70, ≥70 years), baseline BMI (<25, ≥25 kg/m²), baseline serum 25(OH)D level (<30, ≥30 nmol/l) or time between last tablet and collection of post-intervention blood sample (≤7, >7 d), we included interaction terms for intervention group by each predictor. The cutoff point of 30 nmol/l was selected based on the Institute of Medicine's 2011 report⁽³⁵⁾. Analyses were repeated within strata of sex, age, baseline BMI, baseline serum 25(OH)D level and timing of blood collection.

Analyses were performed in SAS version 9.2 (SAS Institute, Inc.). All *P* values are two-sided, and we used a statistical significance level of *P* < 0.05.

Sample size. The trial was designed to have adequate power to assess changes in serum 25(OH)D levels⁽³³⁾. The final number enrolled was greater than planned because of an increase in response rate in later waves of recruitment.

Given strong recommendations against their use⁽³⁶⁾, *post hoc* power calculations have not been performed for this secondary analysis of inflammatory markers. This is, however, one of the largest randomised placebo-controlled trials conducted to date that investigates the effect of vitamin D supplementation on inflammatory markers.

Results

Characteristics of participants

We randomised 644 participants, 615 of whom completed the study. Post-intervention blood samples for the analysis of inflammatory markers were available for 613 participants. The median time between the last tablet taken and collection of the post-intervention blood sample was 7 (IQR 6–11) d and, within each treatment group, 93% of people provided the sample within 2 weeks of taking the final tablet. The average age of participants included in this analysis was 72 (SD 7) years, and 46% were women. Baseline characteristics are presented in Table 1. Baseline serum 25(OH)D levels were similar for the three groups (Table 1), whereas average post-intervention serum 25(OH)D levels were highest in the 1500 µg group (78 (SD 20) nmol/l), intermediate in the 750 µg group (64 (SD 17) nmol/l) and lowest (unchanged) in the placebo group (42 (SD 15) nmol/l)⁽³³⁾; *P* values for all pairwise comparisons were <0.0001. The percentage of participants with serum 25(OH)D levels >75 nmol/l also significantly differed between groups (1500 µg group, 54%; 750 µg group, 24%; placebo group, 1%).

Analysis of inflammatory markers

Each of CRP, leptin, adiponectin and LAR had one missing observation, and data were missing for IL-6 and IL-10 in four and twelve cases, respectively. The percentages of observations with a biomarker level below the limit of detection were 1% (CRP), 32% (IL-6), 58% (IL-10), 3% (leptin) and 2% (adiponectin); the percentages did not differ between treatment groups. The 75th percentile IL-6 level was 2.8 pg/ml higher (95% CI 0.4, 5.8 pg/ml) in the 1500 µg group than in the placebo group (75th percentiles: 11.0 *v.* 8.2 pg/ml) (Table 2). There was a somewhat smaller and non-significant difference (in 75th percentile IL-6 levels) between the 750 µg and placebo groups (Table 2). There were no statistically significant differences in median levels of CRP, leptin, adiponectin or LAR, or in 75th percentile levels of IL-10 levels, between the placebo group and either the 750 or the 1500 µg group (Table 2).

There was a significant interaction between the intervention group and age with respect to the association between supplementation and median levels of leptin and LAR (*P* interaction = 0.03 and 0.02, respectively) (online Supplementary Table S1). For people aged <70 years, the median leptin and LAR levels were higher in the 750 µg group and

Table 1. Baseline characteristics of participants by treatment group (Mean values with their standard deviations; percentages)

Variable	Placebo (<i>n</i> 205)		750 µg (<i>n</i> 203)		1500 µg (<i>n</i> 205)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	71.5	6.8	71.6	7.0	71.5	6.8
BMI (kg/m ²)*	27.5	4.7	27.3	4.6	27.2	4.1
Baseline serum 25(OH)D (nmol/l)†	42.0	13.5	41.6	12.7	41.9	14.3
Physical activity (METs/week)	45.7	45.2	46.6	49.1	46.0	52.7
Time outdoors (h/week)	14.0	12.0	15.0	12.7	14.7	12.3
Vitamin D intake (µg/d)	3.1	3.6	3.3	4.8	3.4	4.3
Sex (%)						
Men	54.6		54.7		51.7	
Women	45.4		45.3		48.3	
Alcohol consumption (drinks/week) (%)						
≤1	45.9		42.4		43.9	
2–6	21.0		22.7		27.3	
7–20	25.4		28.6		23.4	
≥21	7.8		6.4		5.4	
Smoking status (%)						
Never smoker	57.1		58.1		60.0	
Ex-smoker	38.5		35.5		36.1	
Current smoker	4.4		6.4		3.9	
Self-reported health status (%)						
Good	85.9		88.2		86.8	
Fair/poor	14.1		11.8		13.2	

25(OH)D, 25-hydroxyvitamin D; MET, metabolic equivalent task.

* Missing data for one participant in the placebo group.

† Missing data for one participant in the 1500 µg group.

Table 2. Comparison of inflammatory markers following 12 months of monthly supplementation with placebo, or 750 or 1500 µg vitamin D₃: quantile regression* (Medians, interquartile ranges, *b* values and 95 % confidence intervals)

Variable	Placebo (<i>n</i> 205)		750 µg vitamin D ₃ (<i>n</i> 203)				1500 µg vitamin D ₃ (<i>n</i> 205)				<i>P</i> †	<i>P</i> trend‡
	Median	IQR	Median	IQR	<i>b</i>	95 % CI	Median	IQR	<i>b</i>	95 % CI		
CRP (mg/l)§	1.3	0.7–3.4	1.5	0.8–3.2	0.1	–0.3, 0.5	1.4	0.8–3.4	0.1	–0.3, 0.6	0.80	0.58
Leptin (ng/ml)§	8.2	3.1–17.7	8.1	2.9–16.6	–0.1	–2.8, 2.1	8.7	4.0–15.4	0.5	–2.4, 2.7	0.89	0.76
Adiponectin (µg/ml)§	13.6	8.0–20.7	12.8	7.9–19.5	–0.9	–3.0, 1.4	12.6	7.8–19.6	–1.0	–2.7, 1.0	0.63	0.37
LAR§	0.6	0.3–1.5	0.6	0.3–1.5	0.1	–0.2, 0.2	0.7	0.3–1.4	0.1	–0.1, 0.3	0.64	0.38
IL-6 (pg/ml)¶	4.0	1.6–8.2	4.5	1.6–9.6	1.4	–1.4, 4.5	4.6	1.6–11.0	2.8	0.4, 5.8	0.24	0.10
IL-10 (pg/ml)¶¶	2.0	2.0–4.2	2.0	2.0–5.3	1.0	–1.2, 4.2	2.0	2.0–3.8	–0.3	–2.3, 2.2	0.55	0.68

IQR, interquartile range; CRP, C-reactive protein; LAR, leptin:adiponectin ratio.

 * For CRP, leptin, adiponectin and LAR, quantile regression coefficients (denoted *b*) represent the estimated difference in medians between the actively supplemented group (750 or 1500 µg) and the placebo group. For IL-6 and IL-10, *b* represents the estimated difference in 75th percentiles.

 † *P* value from the Wald test for intervention group treated as a categorical predictor (with 2 df), with the placebo group used as the reference level.

 ‡ *P* trend from the Wald test for intervention group treated as a continuous variable (1 df) with values 0, 1 and 2 corresponding to the placebo, 750 and 1500 µg groups, respectively.

§ Missing data for one participant in the placebo group.

¶ Missing data for three participants in the placebo group and one participant in the 750 µg group.

¶¶ Missing data for four, three and five participants in the placebo, 750 and 1500 µg groups, respectively.

lower in the 1500 µg group compared with the placebo group. The opposite pattern was observed for those aged at least 70 years. There were no other significant interactions.

Discussion

Despite large differences in serum 25(OH)D levels between the three groups after 12 months of vitamin D supplementation, we found little evidence of an effect of supplementation on levels of cytokines and adipokines, with the possible exception of IL-6. The significantly higher 75th percentile IL-6 level in the 1500 µg group compared with the placebo group was in contrast to our expectations.

There have been at least ten RCT examining the effect of vitamin D supplementation on IL-6 or IL-10 concentrations. Of the RCT that included participants either without a specific health condition^(23–26) or that only included overweight or obese participants^(27–29), the results for IL-6 and IL-10 were all essentially null. In one of these, there was a suggestion of a greater change in median IL-10 levels in the supplemented group (–70 %) than in the placebo group (–50 %), but the difference was not significant after adjustment for multiple testing⁽²⁶⁾. Possible explanations for these null results include that the sample sizes were too small, the duration or dose of supplementation was insufficient to elicit a response or that the people studied had adequate vitamin D status at baseline.

However, for almost all of these trials, supplementation significantly increased serum 25(OH)D levels^(24–29), and in two trials^(25,28) participants had low baseline 25(OH)D levels.

It is not clear why, in the present study, the upper quartile IL-6 level was greater for those receiving the highest dose of vitamin D supplementation than in the placebo group, and it is possible that this is a chance finding. It is also possible that vitamin D supplementation might be detrimental at higher doses. However, in addition to having pro-inflammatory effects, there is also evidence that IL-6 exhibits anti-inflammatory properties^(37,38). Further, in some *in vitro* studies using human cells, treatment with 1,25(OH)₂D₃ increased IL-6 production by differentiated adipocytes⁽⁸⁾, and induced a subpopulation of IL-6-producing T cells when administered to T lymphocytes⁽³⁹⁾.

Results from trials measuring CRP have been mixed, with no consistent evidence of any clinically relevant effect. In many RCT in which participants did not have a specific disease, CRP levels did not significantly change^(23–25,27–29,40). Vitamin D supplementation was associated with a significant reduction in circulating concentrations of CRP in healthy pregnant women⁽¹⁷⁾ and in patients with a history of myocardial infarction⁽¹⁸⁾, but CRP levels increased in another trial among people who were overweight or obese⁽⁴¹⁾.

With respect to the adipokines, some observational studies have reported positive associations between serum 25(OH)D level and adiponectin levels^(12,13), with one study using a Mendelian randomisation approach to demonstrate that the association was possibly causal⁽¹⁴⁾. In contrast, an inverse correlation has been observed between serum 25(OH)D levels and circulating concentrations of leptin⁽¹¹⁾. Trial data concerning adipokines are scarce and those that have been conducted had small sample sizes^(22,28,42). To the best of our knowledge, only one trial has reported a significant increase in adiponectin levels following vitamin D supplementation⁽²²⁾. The study was restricted to participants with type 2 diabetes, and the end-of-study adiponectin levels reported were much lower than in our participants. We found no difference in levels of either adipokine between placebo and active supplementation groups. The significant interaction between the intervention and age with respect to leptin and LAR levels is mostly likely a chance finding, as there was no dose–response relationship in stratified analyses.

Our results were largely null. For a given biomarker, this means that either that the effect due to supplementation was too small to be of biological importance or that the sample size was insufficient to detect a small but potentially important effect. The CI provide a range of plausible values for the actual effect size.

Cytokine regulation may be dependent on the serum 25(OH)D levels achieved by supplementation. The authors of a study that showed that IL-6 production by human monocytes was inhibited by treatment with ≥ 75 nmol/l (but not 37.5 nmol/l) 25(OH)D₃, concluded that maintaining a serum 25(OH)D level ≥ 75 nmol/l was necessary for optimal anti-inflammatory effects⁽⁴³⁾. A small observational study of young adults (25–42 years) also noted that levels of selected pro-inflammatory cytokines were higher in those with serum 25(OH)D levels ≤ 80 nmol/l when compared with people with higher levels⁽⁴⁴⁾. Approximately one half of

our participants in the 1500 μ g group achieved a post-intervention serum 25(OH)D level ≥ 75 nmol/l, compared with just 1% in the placebo group. Thus, for comparisons between the 1500 μ g group and the placebo group, it seems unlikely that our mostly null results are because of inadequate serum 25(OH)D levels following supplementation.

This study has some limitations. Diet influences systemic inflammation, and thus it was not ideal to have used non-fasting blood samples. Although this has the potential to influence our summary statistics, the randomisation eliminates any risk of biased estimates of treatment effect that might have otherwise resulted. Because we did not measure baseline levels of inflammatory markers, we could not assess baseline equivalence and could not analyse changes in levels. The lack of baseline data is extremely unlikely to have led to any bias in these results. The trial groups were extremely well balanced at baseline⁽⁴⁵⁾, and it would be extraordinary if the distribution of any biomarkers differed before the intervention. Thus, any differences after the intervention are an indication of change from baseline. We used a monthly bolus dose of vitamin D, which some have argued might confer suboptimal benefits (for some endpoints) when compared with daily dosing^(46–48). However, two studies found that daily or monthly dosing did not produce significantly different serum 25(OH)D levels after 2 months⁽⁴⁹⁾ or 1 year⁽⁵⁰⁾ of vitamin D₃ supplementation. Given the relatively long half-life of vitamin D and 25(OH)D⁽⁵¹⁾, variability in timing of post-intervention blood samples should also not be responsible for our largely null findings. Although non-differential follow-up could introduce bias, our extremely high completion rate (96%) means that any bias would be negligible and would not have materially influenced the results. Caution should also be exercised in generalising our results, as our population was older and predominantly (95%) Caucasian.

Our study also has strengths. It is one of the largest randomised placebo-controlled trials with measurement of inflammatory markers conducted to date, and, importantly, our participants were recruited from the general population. Consequently, our results may be more relevant to the general elderly population than those from studies focusing on people with specific diseases. The duration of our intervention and the doses administered were sufficient to elicit measurable increases in serum 25(OH)D levels, and our study had high rates of retention and adherence (96% of participants reported taking at least ten of the twelve study tablets).

With the possible exception of IL-6, we found little evidence of an effect of vitamin D supplementation on cytokine or adipokine levels. These results highlight the uncertainty surrounding the effectiveness of vitamin D supplementation in improving inflammatory biomarker profiles and the need to better understand the effects of vitamin D supplementation before recommending that it is routinely used in the general population.

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Supplementary material

For supplementary material/s referred to in this article, please visit <http://dx.doi.org/10.1017/S0007114515002366>

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