Evolution of Serum 25OHD in Response to Vitamin D₃ Fortified-Yogurts Consumed by Healthy Menopausal Women: A Six-Month Randomized-Controlled Trial Assessing the Interactions between Doses, Baseline Vitamin D Status and Seasonality

Jean-Philippe Bonjour ¹,*, Flore Dontot-Payen ², Emilien Rouy ², Stephane Walrand ³ and Brigitte Rousseau ²

¹ Division of Bone Diseases, University Hospitals and Faculty of Medicine, Geneva, Switzerland (JPB),
² Groupe de Recherche Nutritionnelle, Yoplait, 150 rue Gallieni, 92641 Boulogne, France (FD-P, ER, BR).
³ INRA, UMR 1019, UNH, CRNH Auvergne, 63000 Clermont-Ferrand, France (SW)

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Email addresses from the authors:
Jean-Philippe Bonjour: jean-philipe.bonjour@unige.ch
Flore Dontot-Payen: flore.dontot@genmills.com
Emilien Rouy: emilien.rouy@gmail.com
Stéphane Walrand: stephane.walrand@clermont.inra.fr
Brigitte Rousseau: brigitte.rousseau@genmills.com

*Corresponding Author and person to who reprint request should be addressed:
Jean-Philippe Bonjour
Division of Bone Diseases
Geneva University Hospitals and Faculty of Medicine
Rue Gabriel Perret-Gentil 4
CH – 1211 Geneva 14, Switzerland
Phone:+4122 382 99 60
Fax:+4122 382 99 73.
E-mail: jean-philippe.bonjour@unige.ch
Funding source: Yoplait France, 150 Rue Galliéni, 92641 Boulogne Cedex, France and General Mills, 1 General Mills Blvd., Minneapolis, Minnesota 55426, USA.

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ABSTRACT

A 24 week-controlled trial was conducted in menopausal women (mean age:61.5) to assess serum 25-hydroxyvitaminD (s25OHD) evolution in relation to three interdependent determinants: doses of supplemented (Suppl.) vitamin D₃ (VitD₃); baseline status; seasonality. Participants were randomized into 3 groups (Gr): Gr.Suppl.0, time-controls maintaining dietary habits; Gr.Suppl.5 and Gr.Suppl.10 consuming one and two 125 g servings of VitD₃-fortified yogurts with 5 and 10 µg daily doses, respectively. The 16 intervention-weeks lasted from early-January to mid-August, the 8 follow-up-weeks from late-August to mid-October. Before enrollment, subjects were randomized into two s25OHD strata: “Low stratum (LoStr)”: 25-50 nmol/L; “High stratum (HiStr)”: >50-75 nmol/L. All enrolled participants remained compliant until study end: Gr.Suppl.0 (n=45), Gr.Suppl.5 (n=44) Gr.Suppl.10 (n=44). Over the 16 intervention and 8 follow-up weeks, s25OHD increased in both supplemented groups, more in Gr.Suppl.10 than Gr.Suppl.5. The constant rate of s25OHD per supplemental VitD₃ microgram was greater in LoStr than HiStr. s25OHD increase was greater with late (mid-March) than early (mid-January) inclusion. In conclusion, this randomized trial demonstrates: -a dose-dependent s25OHD improvement related to fortified yogurt consumption; -an inversely baseline-dependent increase in s25OHD; -a seasonal effect that highlights the importance of vitamin D₃ supplementation during winter, even at 5µg/d, in healthy menopausal women.

KEY WORDS: nutritional intervention; menopausal women; vitamin D₃-fortified yogurts; serum 25OHD dose response; seasonality interaction
1. Introduction

Improving the vitamin D status of the general population is recognized as an important public health commitment [1-5]. The vitamin D status can be assessed by the measurement of its main circulating metabolite, namely 25-hydroxyvitamin D (25OHD). Among adults, the risk of vitamin D insufficiency (25OHD < 50 nmol/L), even deficiency (25OHD < 25 nmol/L) increases with aging [2,6]. The greater risk appears to be in elderly population, particularly those living in institutions, who for various reasons have a limited access to sun exposure that is not compensated by an adequate vitamin D intake [7]. Nevertheless, younger populations, such as menopausal women in their late sixties and early seventies also include a certain number of individuals with serum 25OHD between 25 and 50 nmol/L, a status that corresponds to vitamin D insufficiency [4,8]. The best-documented outcome of this inadequate supply is the risk of fragility fractures that augments with advancing age [4,8-10].

The limited vitamin D supply provided by most foods usually consumed in industrialized countries requires an alternative strategy for preventing the development of insufficient or even deficient status of this micronutrient in the general population. Fostering sun exposure might theoretically represents an alternative strategy, since ultraviolet radiation of 290-315 nm wavelength (UVB) stimulates the cutaneous photosynthesis of vitamin D₃ (cholecalciferol) from 7-dehydrocholesterol [11]. However, this potential alternative is far from being straightforward. Indeed, the production of vitamin D₃ by the skin is dependent upon several factors including seasonality, geographical location (latitude, altitude), ozone layer, cutaneous melanin pigment, aging, obesity and body mass index (BMI), let alone the widespread practice of sun avoidance and/or use of protection creams to curtail skin cancer risk [8,11-15]. Therefore, taking all these various determinants into account, it remains a challenge to recommend the appropriate sunlight exposure “dose“ in order to achieve a sufficient vitamin D status without increasing skin cancer risk [15-17].
It appears somewhat less challenging to determine the sufficient amount of vitamin D3 whether taken orally in pharmaceutical preparations or in fortified foods. Nevertheless, the impact of vitamin D3 intake on its status depends upon several factors including: i) dosage; ii) baseline 25OHD level; iii) season of the year. These three determinants have been identified in several observational or interventional studies [2,5,8,12-14,18-27]. How these three determinants quantitatively interact remains to be documented in a single prospective study enrolling well-characterized subjects.

In order to test these assumptions we designed a twenty-four week-randomized controlled trial to quantitatively assess the evolution of serum 25OHD in response to two amounts of vitamin D3 as supplied in fortified yogurts in a cohort of healthy menopausal women. The study was beforehand designed to highlight the interaction of the serum 25OHD response with the baseline vitamin D status and the influence of seasonality.
2. Materials and Methods

2.1. Ethical aspects

The study was carried out in accordance with the “Declaration of Helsinki” as modified in Fortaleza (Brazil) in October 2013, and the recommendations on Good Clinical Practice (ICH E6) and any applicable local regulatory requirements. The study started upon receipt of the approval of both the Ethics Committee (“Comité de Protection des Personnes” and the French Health Authorities (Agence nationale de sécurité du médicament et des produits de santé, ANSM).

2.2. Participants

They were recruited among community-dwelling women in the Auvergne-Rhône-Alpes region in France. Only study-specific recruitment tools approved by the European Community (EC) were used. These recruitment tools included: volunteers’ database from the General Clinical Research Center (GCRC), Eurofins-Optimed (38610 Gières France); regional newspapers advertisement with specific press inserts; radio spots and broadcast messages; posters; mailing; GCRC recruitment website. 288 volunteers expressed interest to participate. The screening procedure occurred within 3 weeks before the intervention. 140 met the study design criteria and were enrolled between January 7 and April 22, 2015.

2.3. Inclusion criteria

They were as follows: women with menopause for \( \geq 5 \) years, aged from 55 to 75 years; informed consent obtained in conformity with the European Directive and French Code of Public Health; body mass index (BMI) ranging from 18 to 28 kg/m\(^2\).

2.4. Exclusion criteria

Use of any form of supplemental vitamin D during the six months preceding the trial, taken as pharmaceutical preparation or through the intake of fortified foods such as milk, dairy products, oil, tofu; functional disability or confinement to bed; concomitant bone
diseases or any illness affecting calcium-inorganic phosphate (Ca-Pi) metabolism such as primary hyperparathyroidism, osteoporotic fracture during the year preceding the study, chronic gastrointestinal disease, chronic renal failure, hepatic and cardiac failure or cancer; treatment during the last 6 months for osteoporosis or other bone diseases, including pharmaceutical agents such as bisphosphonates, raloxifen, teriparatide, strontium ranelate and denosumab; current glucocorticoid treatment; ongoing hormonal replacement therapy; lactose intolerance or any substantial food allergy; participation in a clinical trial during the three months preceding the entry into the study.

2.5. Design and conducted trial

The study was a randomized open-label controlled trial conducted in one single General Clinical Research Center (GCRC) localized at Gières, (Isère Departement of the Auvergne-Rhône-Alpes Region) France.

The aim was to evaluate during 16 weeks the effects of a daily consumption of one or two yogurts fortified in vitamin D and calcium on the evolution of 25OHD. One yogurt pot of 125 g provided: 5 µg of vitamin D₃; 400 mg of calcium; 5 g of protein; 88 kcal of energy.

A randomization list was used by the GCRC to distribute the participants into 3 groups:

- Gr.Suppl.0: parallel time controls that were recommended not to change their dietary habits during the 24-weeks study.
- Gr.Suppl.5: consumption of 1 yogurt per day during 16 weeks followed by 8 weeks without product.
- Gr.Suppl.10: consumption of 2 yogurts per day during 16 weeks followed by 8 weeks without product.

Ambulatory visits at the clinical research center were scheduled at inclusion or baseline (BSL) and after weeks 4 (WK4), 8 (WK8), 12 (WK12), 16 (WK16), 24 (WK24). At each visit, a physical examination was performed including body weight, waist circumference,
blood pressure, heart rate measurements and blood sampling. The participants handed over a diary with information on compliance, product acceptability questionnaire, sun exposure and recording of any adverse event (see below).

2.6. Compliance and acceptability evaluation

The participants randomized to consume one (n=44) or two yogurts (n=44) per day were asked to complete questionnaires about the compliance and acceptability of the product. The compliance was noted each day by the participants in their diary. Further, they were asked to keep the yogurt caps. The acceptability was assessed at WK 4, 8, 12 and 16 following the onset of intervention. A scale from 0 to 10 (do not agree at all = 0; to completely agree = 10) had to be completed in response to the following questions: a) “the dairy product has a pleasant taste? “; b) “its size is suitable for my appetite? “; c) “consumption at a rate of 2 pots per day is not too restricting, i.e. does not limit the food intake at lunch and dinner? “; d) “I am not tired of consuming it? “. The responses were noted by the investigator in the appropriate section of the case report form.

2.7. Sun exposure and vitamin D supplies

Participants were asked to limit daily sun exposure with bare arms or legs to no longer than 20 minutes and not to attend tanning center. The subjects had to report whether at any time of the 24 investigation weeks they spent more than 20 minutes daily with uncovered arms exposed to the sun or traveled to regions with UV exposure greater than in the investigation area. The dietary vitamin D supplies were assessed using a questionnaire on the consumption of vitamin D containing foods [28] and filled in at BSL, WK16 and WK 24.

2.8. Biochemical analysis

The blood samples were collected in the morning after an overnight fast and stored at -70° C until analysis. Serum 25OHD was determined by two successive methods. For the screening samples, serum 25OHD was measured by an automated electro-luminescence
immunoassay (ECLIA) using a Cobas 6000, Roche Diagnostics, Rotkreuz, Switzerland. Then, in all samples collected from BSL to WK24, and thus taken into account in the statistical analysis of the results, serum 25OHD was measured by enzyme-linked immunoabsorbent assay (ELISA, Promokine, Heidelberg, Germany) on a Bio-Rad Microtech Microplate Reader (Hercules, CA, USA). The intra-assay and inter-assay variations were for both assays less than 7.0%. All analytical measurements from BSL to WK24 were run together. Each sample was measured in duplicates.

The option to shift from ECLIA used at screening to ELISA as applied for the whole trial, was warranted by the greater sensitivity of the second method. This shift resulted in an expansion of the serum 25OHD range from 21-76 (screening) to 7-74 (BSL) nmol/L, as determined in the 133 samples collected in all participants included and compliant during the 24 week trial.

2.9. Statistical analysis

Determination of the sample size was estimated in order to highlight a difference in serum 25OHD of 7.5 nmol/L between the yogurt-consuming groups (active) and the control group (primary outcome). Taking into account a serum 25OHD standard deviation (SD) of 10 nmol/L, in order to achieve a power of 90% and a 2-sided-\( \alpha \) of 5%, the overall number of subjects to be included was estimated at 105, i.e. 35 per group. With an anticipated dropout rate of 25%, 140 participants were eventually randomized, i.e. 46, 47 and 47 in Gr.Suppl.0, Gr.Suppl.5 and Gr.Suppl.10, respectively. Seven subjects dropped out, before the onset of the intervention: 1, 3 and 3 in Gr.Suppl.0, Gr.Suppl.5 and Gr.Suppl.10, respectively. The statistical analysis was applied to all included and compliant subjects (n=133) and in addition to two subgroups stratified according to the serum level of 25OHD from 25 to 50 nmol/L and from \( \geq 50 \) to 75 nmol/L as measured in the screening samples. This stratification generated the following two subpopulations: low stratum (LoStr), n= 53; high stratum (HiStr), n=80
(secondary outcome). Over the 16 intervention weeks and the following 8 weeks after treatment discontinuation, the differences in the time course and the tested products for serum 25OHD were evaluated by repeated-measure analysis of variance (RANOVA) with adjustment by the Tukey’s test. Over the first 8 weeks of intervention (from BSL to WK8), the rate constant of changes in serum 25OHD per µg of consumed vitamin D₃ (nmol/L . µg vitamin D₃⁻¹) was calculated for both Gr.Suppl.5 µg and Gr.Suppl.10 µg (secondary outcome). The influence of seasonality was assessed by dichotomizing the three randomized groups (Gr.Suppl.0, Gr.Suppl.5 and Gr.Suppl.10) into “Early“ (or “winter set“) and “Late“ ( “spring set“) according to the date of their enrollment (secondary outcome). The absolute values at BSL, WK4, WK8 and WK16 as well as the changes from BSL to WK8 and to WK16 between “Early“ and “Late“ enrollment were compared by variance analysis for each of the three randomized experimental groups. Statistical analyses were performed using SAS software, version 9.3 (SAS Insitut Inc, Cary, NC, USA).
3. Results

3.1. Demographic characteristics at baseline

There was no significant difference between the three randomized groups in relation to either age or anthropometric variables (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Gr.Suppl.0</th>
<th>Gr.Suppl.5</th>
<th>Gr.Suppl.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.6 (0.8)</td>
<td>60.4 (0.6)</td>
<td>61.4 (0.8)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.1 (1.1)</td>
<td>64.0 (1.4)</td>
<td>63.9 (1.1)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.0 (0.8)</td>
<td>161.5 (0.8)</td>
<td>160.9 (0.9)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5 (0.4)</td>
<td>24.5 (0.5)</td>
<td>24.7 (0.4)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>86.8 (1.1)</td>
<td>85.3 (1.4)</td>
<td>85.9 (1.2)</td>
</tr>
</tbody>
</table>

Values are means (SE).
Gr.Suppl.0: No vitamin D₃-fortified yogurt consumption. Recommendation to maintain dietary habits during 24 weeks.
Gr.Suppl.5: One vitamin D₃-fortified yogurt/d during 16 weeks followed by 8 weeks without product.
Gr.Suppl.10: Two vitamin D₃-fortified yogurts/d during 16 weeks followed by 8 weeks without product.
There was no statistically significant difference (overall ANOVA) between the 3 groups for any of the five characteristics.

3.2. Serum 25OHD

The evolution of the serum 25OHD level in the 133 participants during the 16 intervention weeks (BSL to WK16) and 8 weeks after treatment discontinuation (WK24) is presented in Table 2.
Table 2. Evolution of serum 25OHD in the three randomized groups

<table>
<thead>
<tr>
<th></th>
<th>Gr.Suppl.0</th>
<th>Gr.Suppl.5</th>
<th>Gr.Suppl.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>45</td>
<td>44</td>
<td>44</td>
</tr>
<tr>
<td>BSL</td>
<td>36.4 (2.4)</td>
<td>36.5 (2.2)</td>
<td>35.9 (2.2)</td>
</tr>
<tr>
<td>WK4</td>
<td>31.8 (2.2)</td>
<td>41.4 (1.8)</td>
<td>44.5 (1.7)</td>
</tr>
<tr>
<td>WK8</td>
<td>36.6 (2.4)</td>
<td>48.8 (1.9)</td>
<td>54.3 (2.5)</td>
</tr>
<tr>
<td>WK12</td>
<td>38.7 (2.6)</td>
<td>51.0 (2.5)</td>
<td>55.4 (2.4)</td>
</tr>
<tr>
<td>WK16</td>
<td>44.1 (2.7)</td>
<td>54.8 (2.4)</td>
<td>59.4 (2.6)</td>
</tr>
<tr>
<td>WK24</td>
<td>49.5 (2.8)</td>
<td>52.6 (2.6)</td>
<td>58.9 (3.0)</td>
</tr>
</tbody>
</table>

25OHD values are means (SE) in nmol/L.
BSL: Baseline visit. WK4 to WK24: Number of weeks elapsed from the baseline visit.
Gr.Suppl.0: No vitamin D₃-fortified yogurt consumption. No change in dietary habits during 24 weeks.
Gr.Suppl.5: One vitamin D₃-fortified yogurt/d during 16 weeks followed by 8 weeks without product.
Gr.Suppl.10: Two vitamin D₃-fortified yogurts/d during 16 weeks followed by 8 weeks without product.
WK16: End of intervention for both Gr.Suppl.5 and Gr.Suppl.10.
WK24: Eight weeks after discontinuation of intervention for both Gr.Suppl.5 and Gr.Suppl.10.
Probability levels for group-by-time interaction (RANOVA) for the evolution of serum 25OHD from BSL to WK16 were:
p=0.0001 for difference between Gr.Suppl.10 or Gr.Suppl.5 and Gr.Suppl.0.
p=0.0417 for difference between Gr.Suppl.10 and Gr.Suppl.5.
Probability levels for the evolution following the discontinuation of intervention i.e. from WK16 to WK24 were:
p=0.0025 and p=0.0321 for differences between Gr.Suppl.0 vs. Gr.Suppl.5 and Gr.Suppl.10, respectively;
p=0.706 for difference between Gr.Suppl.10 and Gr.Suppl.5.

The baseline level (BSL) was quite similar in the 3 randomized groups. The serum 25OHD level (about 36 nmol/L) was within the insufficiency range, in conformity with the upper limit
of 50 nmol/L that was predetermined as an inclusion criterion. A rapid increase in the serum 25OHD was already significant after 4 weeks of fortified yogurt consumption (Figure 1).

![Figure 1]

**Figure 1**

Change (Δ) of serum 25OHD in nmol/L from baseline (BSL) to week (WK) 4, 8, 12 and 16 after the onset of intervention. 133 healthy menopausal women were randomized into 3 groups: Gr.Suppl.0, who were advised not to change their dietary habits; Gr.Suppl.5 who daily consumed one fortified yogurt that provided 5 µg of vitamin D3 per day. Gr.Suppl.10 who consumed two fortified yogurts that provided 10 µg of vitamin D3 per day. The SE values are written in association with each column. Statistical test by RMANOVA indicates that the differences between Gr.Suppl.5 or Gr.Suppl.10 and Gr.Suppl.0 was significant at \( P=0.0000 \). The difference between Gr.Suppl.5 and Gr.Suppl.10 was significant with \( P=0.0417 \).

The increase was greater in Gr.Suppl.10 than Gr.Suppl.5 (RANOVA: \( P=0.0417 \)). After 8 weeks (WK8) of yogurt consumption, serum 25OHD already amounted to 89.1% (Gr.Suppl.5) and to 94.1% (Gr.Suppl.10) of the level measured after 16 weeks (WK16) of intervention (Table 2). In the control group (Gr.Suppl.0), during the first 8 weeks, serum
25OHD remained stable: 36.4 at BSL and 36.6 nmol/L at WK8 (Figure 1). Thereafter, it significantly rose to reach a level of 44.1 nmol/L at week 16 (WK 16) (Table 2). After discontinuation of the intervention, from WK16 to WK24, the 25OHD level was virtually maintained in both Gr.Suppl.10 (-0.57±1.68 nmol/L, \(p=0.738\)) and Gr.Suppl.5 (-2.20±1.62 nmol/L, \(p=0.182\)) while it significantly rose in Gr.Suppl.0 (+5.53±1.61, \(p=0.0015\)) (Table 2).

As indicated in the legend to Table 2 the evolution from WK16 to WK24 was significantly different between the two groups having consumed vitamin D-fortified yogurts and the control group.

3.3. Serum 25OHD response in relation to baseline level

Based on screening 25OHD levels, the participants were segregated into low stratum (LowStr from 25 to 50 nmol/L, n=53) and high stratum (HighStr \(\geq\) 50 to 75 nmol/L, n=80). The evolution from BSL to WK24 of the absolute serum 25OHD values indicates a dose effect of vitamin D\(_3\)-fortified yogurts in both LowStr and HighStr subgroups (Table 3).
Table 3. Evolution of serum 25OHD in subjects distributed according to their initial vitamin D status

<table>
<thead>
<tr>
<th></th>
<th>Gr.Suppl.0</th>
<th>Gr.Suppl.5</th>
<th>Gr.Suppl.10</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Stratum</td>
<td>High Stratum</td>
<td>Low Stratum</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>BSL</td>
<td>22.0 (1.7)</td>
<td>46.0 (2.3)</td>
<td>26.8 (2.4)</td>
</tr>
<tr>
<td>WK4</td>
<td>19.2 (2.1)</td>
<td>40.1 (2.3)</td>
<td>35.3 (2.2)</td>
</tr>
<tr>
<td>WK8</td>
<td>24.9 (2.5)</td>
<td>44.4 (2.9)</td>
<td>44.2 (2.6)</td>
</tr>
<tr>
<td>WK12</td>
<td>27.7 (3.1)</td>
<td>46.1 (3.2)</td>
<td>45.3 (2.8)</td>
</tr>
<tr>
<td>WK16</td>
<td>33.8 (3.8)</td>
<td>51.0 (3.0)</td>
<td>50.2 (3.1)</td>
</tr>
<tr>
<td>WK24</td>
<td>37.9 (3.3)</td>
<td>57.2 (3.4)</td>
<td>46.0 (3.1)</td>
</tr>
</tbody>
</table>

25OHD values are means (SE) in nmol/L.

BSL: Baseline visit. WK4 to WK24: Number of weeks elapsed from the baseline visit.
The participants were distributed in low or high stratum according to their serum 25OHD screening values, i.e. before the BSL visit.
Low stratum range: 25 to 50 nmol/L. High stratum range: ≥ 50 to 75 nmol/L.
Gr.Suppl.0: No vitamin D₃-fortified yogurt consumption. No change in dietary habits during 24 weeks.
Gr.Suppl.5: One vitamin D₃-fortified yogurt/d during 16 weeks followed by 8 weeks without product.
Gr.Suppl.10: Two vitamin D₃-fortified yogurts/d during 16 weeks followed by 8 weeks without product.
WK16: End of intervention for both Gr.Suppl.5 and Gr.Suppl.10.
WK24: After discontinuation of intervention for both Gr.Suppl.5 and Gr.Suppl.10.

-Probability levels for group-by-time interaction (RANOVA) for the evolution of serum 25OHD from BSL to WK16 were:
Low Stratum: \( p = 0.0001 \) and \( p = 0.0012 \) for difference between Gr.Suppl.10 or Gr.Suppl.5 and Gr.Suppl.0, respectively.
\( p = 0.0346 \) for difference between Gr.Suppl.10µg and Gr.Suppl.5µg.
The changes from baseline to 8 weeks (BSL to WK8) were for Gr Suppl.5: LowStr 17.4 vs. HighStr 8.4 nmol/L (Figure 2). It was for Gr Suppl.10: LowStr, 27.1 vs. HighStr, 13.5 nmol/L; for Gr Suppl.0: LowStr 3.0 vs. HighStr -1.6 nmol/mL (Figure 2). Thus, the absolute increase in serum 25OHD after 8 weeks of intervention was greater in LowStr than in HighStr regardless of the daily vitamin D3 consumed amounts (5 or 10 µg).
Figure 2

Change (Δ) of serum 25OHD in nmol/L from baseline (BSL) to week (WK) 4, 8, 12 and 16 after the onset of intervention. 133 healthy menopausal women were randomized into 3 groups: Gr.Suppl.0µg, who were advised not to change their dietary habits; Gr.Suppl5µg, who daily consumed one fortified yogurt providing 5 µg of vitamin D₃ per day. Gr.Suppl.10µg who daily consumed two fortified yogurts providing 10 µg of vitamin D₃ per day. Based on serum 25OHD values measured in screening samples, the cohort was divided into two strata: low stratum, from 25 to 50 nmol/L; high stratum, from >50 to 75 nmol/L. The statistical significances by RMANOVA indicate that the differences between Gr.Suppl.5 or Gr, Suppl.10 and Gr.Suppl.0 for low stratum were significant at \( P=0.0012 \) and \( P=0.0001 \), respectively. For high stratum, the corresponding significances were \( P=0.0020 \) and \( P=0.0001 \), respectively. The difference between Gr.Suppl.5 and Gr, Suppl.10 was significant \( P=0.0346 \) in low stratum, but not in high stratum, \( P=0.416 \). The influence of the baseline serum 25OHD level on the serum 25OHD progressive increase during the intervention was significantly greater in low than high stratum \( (P=0.0001) \).
3.4. Serum 25OHD rate constant

The rate constant was calculated based on the increase in serum 25OHD per µg of vitamin D₃ consumed from BSL to WK8. It was not significantly different with 5 µg of vitamin D₃ compared to 10 µg (Figure 3). It was 2.3 (Gr.Suppl.5: 2.18/0.96) and 2.9 (Gr.Suppl.10: 2.02/0.70) fold greater in LowStr than in HighStr (Figure 3).
Figure 3

Changes of serum 25OHD during the first eight weeks following the consumption of one or two vitamin D3-fortified yogurts that increased the intake of vitamin D3 up to 5 and 10 µg per day, respectively. Columns indicate the changes (= rate constant) of serum 25OHD in nmol/L per 1 µg of supplemental vitamin D3. The totality (ALL) of participants were separated into high stratum and low stratum according to the values of serum 25OHD as measured in screening samples: > 50 to 75 and 25 to ≤ 50 nmol/L, respectively. Number of subjects: All, 44 and 44; High Stratum, 25 and 28; Low Stratum, 19 and 16, in Gr.Suppl.5 and Gr.Suppl.10 µg of vitamin D3 per day, respectively. Statistical evaluation by ANOVA between Gr.Suppl.5 and Gr.Suppl.10: ALL, P=0.488; High stratum, P= 0.452; Low stratum, P= 0.794. Difference between High stratum and Low stratum: P= 0.0006.
3.5. Serum 25OHD seasonal effect

The 133 participants were enrolled from early January 6 to late April 2015. In order to assess whether serum 25OHD was influenced by the enrollment time, we analyzed the evolution of the vitamin D status after dichomization of the three investigated groups in Early (BSL in January) compared to Late (BSL in March) enrollment (Table 4).
Table 4. Influence of sampling date on serum 25OHD evolution in healthy menopausal women supplemented with vitamin D₃ at 5 (Gr.Suppl.5) or 10 µg/d (Gr.Suppl.10) as compared to time controlled non-supplemented participants (Gr.Suppl.0)

<table>
<thead>
<tr>
<th>Sampling Time</th>
<th>Gr.Suppl.0</th>
<th>Gr.Suppl.5</th>
<th>Gr.Suppl.10</th>
<th>p.0</th>
<th>p.5</th>
<th>p.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Datevalue BSL: Range</td>
<td>42010-42039</td>
<td>42044-42116</td>
<td>42010-42044</td>
<td>42044-42116</td>
<td>42010-42044</td>
<td>42046-4116</td>
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<td><strong>25OHD BSL</strong></td>
<td><strong>35.1 (3.0)</strong></td>
<td><strong>37.6 (3.6)</strong></td>
<td><strong>36.8 (3.0)</strong></td>
<td><strong>36.3 (3.3)</strong></td>
<td><strong>38.1 (3.2)</strong></td>
<td><strong>33.6 (3.1)</strong></td>
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<td><strong>41.5 (3.7)</strong></td>
<td><strong>48.4 (2.3)</strong></td>
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<td><strong>51.6 (3.9)</strong></td>
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<td>09/01/2015</td>
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<tr>
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<td><strong>42.9 (3.3)</strong></td>
<td><strong>55.8 (4.2)</strong></td>
<td><strong>49.4 (2.8)</strong></td>
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<td><strong>50.7 (3.2)</strong></td>
<td><strong>67.0 (4.5)</strong></td>
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Serum 25OHD values are means (SE) in nmol/L.
Datevalue = Date stored as text in month/day/year (M/D/Y) converted into a serial number, 1=01/01/1900.
The participants were divided in “Early“ or “Late“ according to their serum 25OHD sampling dates at baseline (BSL).
The next measurements were made after 8 and 16 weeks (WK) of intervention and 8 weeks after discontinuation of intervention (WK24).
Gr.Suppl.0: No vitamin D₃-fortified yogurt consumption. No change in dietary habits during 24 weeks.
Gr.Suppl.5: One vitamin D₃-fortified yogurt/d during 16 weeks followed by 8 weeks without product.
Gr.Suppl.10: Two vitamin D₃-fortified yogurts/d during 16 weeks followed by 8 weeks without product.
p.0, p.5, p.10: Probability level in serum 25OHD differences between samplings Early and Late for Gr.Suppl.0, 5 and 10 µg, respectively.
At BSL, in each randomized groups there was no significant difference in serum 25OHD between Early and Late enrollment (Table 4). In the control group (Gr.Suppl.0), the differences between Early vs. Late enrollment were 9.9 nmol/L at WK8 ($p=0.041$) and 15.4 nmol/L at WK16 ($p=0.003$) (Table 4). In Gr.Suppl.5µg, the increase in serum 25OHD was similar in Early and Late subgroups (Table 4). In Gr.Suppl.10, there was a progressive greater rise of serum 25OHD in Late as compared to Early enrollment with a difference of 10.4 nmol/L ($p=0.042$) after 4 months (WK16) of intervention. Two months after discontinuation of fortified yogurt consumption with an amount of 10 µg/d, a greater 25OHD level was recorded with Late vs Early enrollment (WK24 +16.3 nmol/L $p=0.005$) (Table 4). The interaction between the vitamin D$_3$ ingested amount and the enrollment time is reflected by the changes in Late vs. Early subgroups after 8 (WK8) and 16 (WK16) weeks of intervention (Figure 4). The 25OHD changes measured in the parallel time-controlled group (Gr.Suppl.0) from BSL to WK8 and WK16 illustrate the influence of the enrollment time (Early: mid-January; Late: mid-March) in absence of any vitamin D$_3$ supplementation (Figure 4).
Figure 4

Changes in serum 25OHD after 8 and 16 weeks (WK) of intervention. The three randomized groups were each divided into two subgroups according to the enrolment dates which were on the average: “Early”, January 16-18, 2015; “Late”, March 13-17, 2015. The figure illustrates the interactions between the sampling date (season effect) and the amount of vitamin D₃ (dose effect) as consumed from fortified yogurts that provided daily either 5 µg (Gr.Suppl.5) or 10 µg (Gr.Suppl.10), and compared to a time-controlled group (Gr.Suppl.0). The SE values are written in association with each column. See Table 4 for the corresponding absolute values, the number of subjects in each subgroup as well as the probability level for the differences in serum 25OHD between “Early” and “Late” for Gr.Suppl.0, 5 and 10 µg of vitamin D₃.
3.6. Evolution of anthropometric variables

Over the 16 weeks of intervention, no significant change was observed in body weight, BMI and waist circumference.

3.7. Compliance and acceptability

Overall, the large majority of the participants remained compliant over the 16 intervention weeks with no significant difference between the consumption of 1 and 2 dairy products per day. The tested product, whether consumed at a daily rate of one or two fortified yogurts, were well accepted in terms of taste, with no negative feeling with respect to the dairy product size that may reduce the appetite and thereby limit the food intake.
4. Discussion

4.1. Main results

In one single randomized clinical trial carried out in menopausal women, this report highlights three interdependent determinants of the response to vitamin D₃-fortified foods. It provides information on the impact of vitamin D₃ fortified dairy taken at two dose levels, as compared to a time-controlled group. This study documents the quantitative influence of the baseline vitamin D status. The calculation of the constant rate of serum 25OHD increment related to the amount of additional vitamin D₃ further underscores the influence of the baseline status on the efficiency of supplementation. Finally, this study emphasizes the distinct impact of two oral amounts of vitamin D₃ in relation to the period of the year during which the intervention is conducted.

4.2. Relation to supplemental doses

The kinetics of the increment in serum 25OHD shows a distinct response between the two supplemental doses of vitamin D₃ (Table 2 and Figure 1). According to the 2011 report from the Institute of Medicine (IOM) on the “Dietary Reference Intake” (DRI) [4], a serum 25OHD level of 40 nmol/L meets the needs of approximately half the population. It corresponds to the median population requirements or the “Estimated Average Requirements (EAR)“. The level of ≥ 50 nmol/L meets the needs of at least 97.5% of the population, it conforms to the “Recommended Dietary Allowance“ (RDA). These two threshold values were primarily based on bone health outcomes [4]. In our study, the baseline serum 25OHD of approximately 36 nmol/L (Table 2) fell within the insufficiency range. Following the consumption of the vitamin D₃ fortified yogurts, the serum 25OHD crossed the sufficiency threshold of 50 nmol/L earlier in time with a dose of 10 µg/d (54.3 nmol/L after 8 weeks) than with a dose of 5 µg/d (54.8 nmol/L after 16 weeks) (Table 2). Thus, the serum 25OHD kinetic response differentiates the two tested amounts of vitamin D₃ better than the absolute level.
attained by the end of the intervention indicates (59.4 vs. 54.8 nmol/L with 10 vs. 5 µg/d after 16 weeks of intervention, \( p = 0.198 \)) (Table 2).

4.3. Relation to initial serum 25OHD level

Previous reports suggested an inverse correlation between baseline vitamin D status and the increase of serum 25OHD in response to either pharmaceutical supplement or food fortification [5]. Thus, in a 12-week Finnish study carried out in forty-nine 65 to 85-year-old women, subjects with lower vitamin D status at baseline responded more efficiently to supplementation than those with more adequate status [29]. In a review and meta-analysis of 16 heterogeneous studies carried out around the world, the treatment effect of vitamin D₃-fortified foods was substantially higher when mean baseline serum 25OHD was \(<50\) rather than \(\geq 50\) nmol/L [30]. The design of our randomized controlled study was pre-specified to test this possible relationship. Our results clearly establish that the lower the baseline vitamin D status is, the higher the absolute increment in serum 25OHD is (Figure 2). The reasons for this inverse relationship remain conjectural. Among possible mechanisms one may tentatively mention: i) the vitamin D status could influence the distribution of circulating 25OHD; ii) the hepatic hydroxylation rate of the cholecalciferol molecule in position 25 could be inversely related to its product; iii) the affinity of the vitamin D binding protein(s) could vary according to the vitamin D status.

4.4. Rate constant of 25OHD increase or vitamin D₃ supplementation efficiency

The concept of rate constant as detailed by Heaney et al. [18] is a useful link between the amount of vitamin D consumed as supplement or fortified foods and the vitamin D status improvement. This value corresponds to an efficiency estimate of the consumed supplemental vitamin D₃ [18]. In a recent systematic review, the rate constant of change in 25OHD expressed as nmol/L per µg of additional vitamin D was calculated from 18 randomized controlled trials [5]. The mean rate constant close to 2 nmol/L per µg of vitamin D [5] was in
agreement with the analysis of 41 studies with an average rate constant of 2.1 [31]. A significant inverse relationship was found between the administered dose of vitamin D and the rate constant of the 25OHD synthesis from its precursor [5]. In our study, there was a non-significant trend for a greater increase in 25OHD per µg of vitamin D in the group consuming 5 rather than 10 µg/d (Figure 3). As discussed above, we observed an important effect of baseline 25OHD on the response to vitamin D₃-fortified dairy. This is reflected by a marked difference in the constant rate measured during the first 8 weeks of intervention; it was more than twice higher in the participants randomized beforehand in the low stratum (25 to 50 nmol/L) than in the high stratum (>50 to 75 nmol/L) (Figure 3).

4.5. Dose-response and seasonality

The magnitude of the effect of vitamin D consumption on its status should reflect both the supplemental dose as well as the season-related evolution of serum 25OHD. Our study run from January to October allows to identify the relative importance of these two factors. Thus, the increase in response to vitamin D₃-fortified yogurts would be substantially over-estimated if one did not take into account the evolution of 25OHD as assessed in our study by monitoring a parallel time-control group. The difference between the control and the two supplemental groups lessened from the onset of the intervention to the end (Table 2 and Figure 1). Most likely, this attenuation of the fortified food effect results from the increased cutaneous production of vitamin D₃. In the control group, serum 25OHD significantly raised from 36.4 to 44.1 nmol/L (+21.2%), i.e. from BSL (mean sampling time mid-February) to WK16 (mean sampling time early-June). It went even further up to 49.5 nmol/L 8 weeks later (WK24, mean sampling time early-August), while in the two intervention groups it tended to slightly decrease following the discontinuation of the vitamin D₃-fortified dairy consumption. Thus, from February to August, without evidence of an increase in the food intake of vitamin D in the control group, the status evolved from insufficiency to reach the lower range of
sufficiency (50 nmol/L) according to the 2011 IOM report [4]. This evolution is in keeping with the seasonality influence on the vitamin D status, as studied in both European countries and the United States [11-14,32]. Quantitatively, serum 25OHD was shown to evolve from 38.5 to 64.0 nmol/L from March to August in community dwelling individuals ≥ 65 years (56.7% men) living in Southern Germany [13]. Whereas the winter value of this German study was very close to ours, the summer level was about 20% less in our trial. This difference may be due, at least in part, to the limitation of sun exposure that was recommended to the participants of our study (see Methods).

The seasonality effect from 36.4 to 49.5 nmol/L in the control group (Table 2) approximately equals the consumption during 8 weeks of fortified dairy providing 5µg/d of supplemental vitamin D₃ that increased the serum 25OHD level from 36.5 to 48.8 nmol/L (Table 2). Nevertheless it is somewhat less than that achieved during the same period, from 35.9 to 54.3 nmol/L, with 10 µg/d of supplemental vitamin D (Table 2). These results corroborate the utility of consuming supplemental vitamin D during the winter season.

4.6. Food fortification for preventing vitamin D insufficiency in the general population

All enrolled subjects remained compliant to the prescribed fortified dairy products, whether consumed as one or two servings per day during 16 weeks. This compliance reflects the good acceptability of providing supplemental vitamin D₃ through the consumption of fortified foods such as soft white cheese or yogurts consumed in studies of shorter duration (for review see [33]).

In terms of public health programs aimed at preventing vitamin D insufficiency, compliance and acceptability are important criteria for achieving a beneficial effect of long term supplementation. Relatively low doses of vitamin D₃ regularly consumed as usual foods offer some advantage over pharmaceutical pills that are either daily taken in small doses but with the risk of low compliance due to medication-related side effects [34] or, alternatively, in
large amounts at monthly or yearly intervals with the non-negligible risk of the occurrence of adverse events. [35]

The results of our randomized trial as conducted in healthy menopausal women are pertinent to the prevention of insufficient vitamin D status in the general population. Quantitatively, the presented evolution of serum 25OHD can be interpreted in relation to the 2011 IOM report that established that a serum 25OHD level of at least 50 nmol/L meets the skeletal health requirements for vitamin D of $\geq 97.5\%$ of the general population. [4] Whether the benefits of vitamin D supplementation would occur only when serum 25OHD are at 75 nmol/L and above is controversial. [3,36] Furthermore, it is even uncertain that such a high level might be required for the adequate management of disease-related conditions such as osteoporosis, chronic kidney disease, hepatic failure, malabsorption syndromes and obesity.[36] In these pathological conditions, dairy or non-dairy food fortification with vitamin D may not be enough to achieve a serum 25OHD level of 75 nmol/L and above [37], particularly when the UVB radiation is limited as recommended to the participants of our trial.

4.7 Strengths and weaknesses

This study has a number of strengths: i) it provides information from a randomized controlled trial on the interaction of three important determinants of the vitamin D status prospectively studied in 133 menopausal women that remained compliant over the 24 weeks of the investigation; ii) the kinetic analysis of the initial eight-week increment of serum 25OHD in response to the consumption of vitamin D$_3$-fortified dairy clearly differentiates the two daily doses tested, namely 5 vs. 10 µg; iii) the study also shows the attenuation of the difference between the two doses of supplemental vitamin D$_3$ during the 16 intervention weeks; iv) it furthermore unequivocally documents the inverse relationship between the baseline vitamin D status and the response to vitamin D$_3$-fortified yogurts; v) it demonstrates the marked interaction between the effect of the vitamin D$_3$ supplementation and the season
dependent onset of the intervention; \textit{vi)} the study highlights the crucial importance of the vitamin D$_3$ supplementation during the winter season, even if as low as 5µg/d, as compared to no supplementation in the control group; \textit{vii)} a positive spin-off of this investigation pertains to its practical utility in terms of public health. Indeed, the results enable to define the efficacious amount of vitamin D$_3$-fortified foods to be consumed by menopausal women according to the season of the year in order to avoid the risk of vitamin D insufficiency.

Weaknesses include: \textit{i)} a randomized open-label controlled trial that implies the absence of a placebo-controlled group. -an estimate of vitamin D supplies based on questionnaires filled in by the participants who provided information on their own sun exposure and frequency of vitamin D-relevant food consumption. \textit{ii)} the arbitrary dichotomization of the participants between low (25 to 50 nmol/L) and high stratum (>50 to 75 nmol/L) based on screening serum 25OHD measurements that used a different analytical method than that applied to the samples collected during the 24 weeks of the trial. Nevertheless, this modification did not attenuate the marked difference in serum 25OHD between the two strata as presented in Table 3 for the absolute values, and in Figure 2 for the evolution changes. \textit{iii)} the cohort was exclusively white and recruited in one single French region. Therefore, the results may not be the same in other ethnic groups, or in populations with different dietary and lifestyle habits and/or living in regions of different latitudes and/or altitudes.

5. Conclusions

This clinical trial in menopausal women provides data on three interdependent determinants of the vitamin D status. It documents a dose-response of vitamin D$_3$-fortified yogurts on the evolution of serum 25OHD during 4 months and the 2 months following the intervention discontinuation. The study unequivocally demonstrates the marked influence of the initial vitamin D status on the absolute increase in serum 25OHD: the lower the baseline status is, the higher the response to a given dose of supplemental vitamin D$_3$ is. The trial was
conducted from January to October of the same year. It enabled to highlight the important influence of the season on the magnitude of the serum 25OHD response to two doses of vitamin D3-fortified yogurts as compared to the maintenance of dietary habits in a time-controlled group. Thus, this randomized controlled trial quantitatively documents the interaction of three key determinants of vitamin D3 status: supplemental doses, initial status and seasonality.
References


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