Evolution of Serum 25OHD in Response to Vitamin D3-Fortified Yogurts Consumed by Healthy Menopausal Women: A 6-Month Randomized Controlled Trial Assessing the Interactions between Doses, Baseline Vitamin D Status, and Seasonality

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Evolution of Serum 25OHD in Response to Vitamin D₃–Fortified Yogurts Consumed by Healthy Menopausal Women: A 6-Month Randomized Controlled Trial Assessing the Interactions between Doses, Baseline Vitamin D Status, and Seasonality

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Introduction

Improving the vitamin D status of the general population is recognized as an important public health commitment [1–5]. This status can be assessed by the measurement of its main circulating metabolite, namely, serum 25-hydroxyvitamin D (s25OHD). Among adults, the risk of vitamin D insufficiency (s25OHD < 50 nmol/L), even deficiency (s25OHD < 25 nmol/L), increases with age [2,6].

The greater risk appears to be in elderly population, particularly those living in institutions and 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 60s and early 70s, also include a certain number of individuals with s25OHD 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, which increases with advancing age [4,8–10].

The limited vitamin D supply provided by most consumed foods in industrialized countries requires an alternative strategy for preventing the development of insufficient or deficient status of this micronutrient in the general population. Fostering sun exposure might theoretically represent an alternative strategy, because ultraviolet radiation of 290- to 315-nm wavelength (ultraviolet B) 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 (VitD₃) by the skin is
dependent on several factors, including seasonality, geographical location (latitude, altitude), ozone layer, cutaneous melanin pigment, aging, obesity, and body mass index (BMI), as well as the widespread practice of sun avoidance and/or use of protection creams to curtail skin cancer risk [8,11–15]. Therefore, taking all of these various determinants into account, it is difficult to recommend the appropriate sunlight exposure "dose" in order to achieve a sufficient vitamin D status without increasing skin cancer risk [15–17].

It is easier 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 (1) dosage; (2) baseline 25OHD level; and (3) season of the year. These 3 determinants have been identified in several observational or interventional studies [2,5,8,12–14,18–27]. How these 3 determinants quantitatively interact remains to be documented in a single prospective study including well-characterized subjects.

In order to test these assumptions, we designed a 24-week randomized controlled trial to quantitatively assess the evolution of 25OHD in response to 2 amounts of vitamin D3 as supplied in fortified yogurt in a cohort of healthy menopausal women. The study was designed to highlight the interaction of the 25OHD response with the baseline vitamin D status and the influence of seasonality.

Materials and methods

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 began 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).

Participants

Participants were recruited among community-dwelling women in the Auvergne–Rhône–Alpes region in France. Only study-specific recruitment tools approved by the European Community were used. These recruitment tools included a volunteer database from the General Clinical Research Center (GCRC), Eurofins-Optimed (Gières, France); regional newspaper advertisements with specific press inserts; radio spots and broadcast messages; posters; mailings; and the GCRC recruitment website. Two hundred eighty-eight volunteers expressed interest in participating. The screening procedure occurred within 3 weeks before the intervention; 140 participants met the study design criteria and were enrolled between January 7 and April 22, 2015.

Inclusion criteria

Inclusion criteria were as follows: women aged between 55 and 75 years with menopause for ≥5 years; informed consent obtained in conformity with the European Directive and French Code of Public Health; and BMI ranging from 18 to 28 kg/m².

Exclusion criteria

Exclusion criteria included the use of any form of supplemental vitamin D whether as pharmaceutical preparation or through the intake of fortified foods such as milk, dairy products, oil, and tofu during the 6 months preceding the trial; functional disability or confinement to bed; concomitant bone disease 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 disease, including pharmaceutical agents such as bisphosphonates, raloxifene, teriparatide, strontium ranelate, and denosumab; current glucocorticoid treatment; ongoing hormonal replacement therapy; lactose intolerance or any substantial food allergy; and participation in a clinical trial during the 3 months preceding entry into the study.

Design and conduct of the trial

The study was a randomized, open-label controlled trial conducted in one single GCRC located in Gières, (Isère Department of the Auvergne–Rhône–Alpes region), France.

The aim was to evaluate the effects of a daily consumption of 1 or 2 yogurts fortified with vitamin D and calcium on the evolution of 25OHD during 16 weeks. One 125-g yogurt pot provided 5 
\mu g VitD₃, 400 mg calcium, 5 g protein, and 88 kcal energy.

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

- Gr.Suppl.0: Parallel time controls that were advised not to change their dietary habits during the 24-week 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), and 24 (WK24). At each visit, a physical examination was performed, including measurement of body weight, waist circumference, blood pressure, and heart rate and blood sampling. Participants provided a diary reporting information on compliance and product acceptability (see below).

Adherence assessment and acceptability evaluation

Participants randomized to consume 1 (Gr.Suppl.5, \( n = 44 \)) or 2 yogurts (Gr.Suppl.10, \( n = 44 \)) per day were asked to complete questionnaires regarding adherence and acceptability of the product. Adherence was noted each day by the participants in a diary. Furthermore, they were asked to keep the yogurt lids. Adherence to or compliance with the intervention in Gr.Suppl.5 and Gr.Suppl.10 was assessed by computing the number of yogurt pot lids returned by the participants at each visit from WK4 to
WK16. Adherence was expressed as the ratio (%) of pots consumed to pots distributed for each period of 4 weeks.

Acceptability was assessed at weeks 4, 8, 12, and 16 following the onset of the intervention. A scale from 0 to 10 (do not agree at all = 0; completely agree = 10) was completed in response to the following questions: “Does the dairy product have a pleasant taste?” “Is its size suitable for my appetite?” “Is consumption at a rate of 2 pots per day too restrictive, that is, does it limit the food intake at lunch and dinner?” “Am I tired of consuming it?” The investigator noted the responses for the last 4 weeks in the appropriate section of the case report form.

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 centers. The subjects had to report whether at any time during the 24 investigation weeks they spent more than 20 minutes daily with uncovered arms exposed to the sun or traveled to regions with ultraviolet exposure greater than in the investigation area. The dietary vitamin D supplies were assessed using a questionnaire on the consumption of foods containing vitamin D [28] and was completed at BSL, WK16, and WK 24.

Biochemical analysis

Blood samples were collected in the morning after an overnight fast. They were stored at −70°C until analysis. s25OHD was determined by 2 successive methods. For the screening samples, s25OHD was measured by an automated electroeluminescence immunoassay (Cobas 6000, Roche Diagnostics, Rotkreuz, Switzerland). Then, for all samples collected from BSL to WK24 and thus included in the statistical analysis of the results, serum 25OHD was measured by enzyme-linked immunosorbent assay (Promokine, Heidelberg, Germany) on a Bio-Rad Microtech Microplate Reader (Hercules, CA). In other words, the screening s25OHD values were measured several days before the baseline measurements.

The intra-assay and interassay variations were less than 7.0% for both assays. All analytical measurements from BSL to WK24 were run together. Each sample was measured in duplicate.

The option to shift from electroeluminescence immunoassay used at screening to enzyme-linked immunosorbent assay used for the whole randomized controlled trial was warranted by the apparent greater sensitivity of the latter method. This shift resulted in an expansion of the s25OHD range from 21–76 (screening) to 7–74 (BSL) nmol/L as determined in the 133 samples collected from all participants included who were compliant during the 24-week trial. The correlation coefficient (r) between the 2 methods was 0.765 as assessed in blood collected at screening and at baseline (mean time interval: 6.4 days) in the 133 subjects who participated in the 24-week randomized study.

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 serum 25OHD standard deviation (SD) of 10 nmol/L, in order to achieve a power of 90% and a 2-sided α of 5%, the overall number of subjects to be included was estimated at 105; that is, 35 per group. With an anticipated dropout rate of 25%, 140 participants were eventually randomized; that is, 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. Statistical analysis was applied to all included and compliant subjects (n = 133) and in addition to 2 subgroups stratified according to s25OHD from 25 to 50 nmol/L and from ≥50 to 75 nmol/L as measured in the screening samples. This stratification generated the following 2 subpopulations: low stratum (LoStr), n = 53; high stratum (HiStr), n = 80 (secondary outcome). Over the 16 intervention weeks and the following 8 weeks after discontinuation, the differences in the time course and the tested products for s25OHD were evaluated by repeated measures analysis of variance (ANOVA) with adjustment by Tukey’s test. Student’s t test was used for changes from BSL to WK16 as well as the Wilcoxon signed rank test whenever the variable was not normally distributed.

Over the first 8 weeks of the intervention (from BSL to WK8), the rate constant of the changes in serum 25OHD per microgram of VitD3 consumed (nmol/L/μg vitamin D3) was calculated for both Gr.Suppl.5 and Gr.Suppl.10 (secondary outcome). The rate constant measurement indicates to what extent added dietary vitamin D3 raises the level of s25OHD compared to baseline. It represents the amount of VitD3 biologically converted to 25OHD as assessed in serum samples at a specific time after the onset of the intervention. It is a quantitative estimate of the efficiency of the intervention. Over the first 8 weeks of the intervention, s25OHD virtually remained constant in Gr. Suppl.0 (see Table 1). This avoided any substraction or addition computed from changes in the time control group.

The influence of seasonality was assessed by dichotomizing the 3 randomized groups (Gr.Suppl.0, Gr.Suppl.5, and Gr. Suppl.10) as early (or winter set) and late (spring set) according to the date of enrollment (secondary outcome). The absolute values at BSL, WK4, WK8, and WK16 as well as changes from BSL to WK8 and to WK16 between early and late enrollment were compared by ANOVA for each of the 3 randomized experimental groups. Furthermore, the differences between the 3 groups from baseline to WK24 in the proportion of subjects displaying a serum 25OHD level ≥50 nmol/L [4] were assessed by chi-square test. Statistical analyses were performed using SAS software, Version 9.3 (SAS Institute Inc., Cary, NC).

Results

Demographic characteristics at baseline

There were no significant differences between the 3 randomized groups in relation to either age or anthropometric variables (Table 1).

s25OHD evolution

The evolution of s25OHD levels in the 133 participants during the 16-week intervention (BSL to WK16) and 8 weeks after
Evolution of serum 25OHD in the 3 randomized groups.

Table 2.

<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 (5.4)</td>
<td>60.4 (4.0)</td>
<td>61.4 (5.3)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.1 (7.4)</td>
<td>64.0 (9.3)</td>
<td>63.9 (7.3)</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>163.0 (5.4)</td>
<td>161.5 (5.3)</td>
<td>160.9 (6.0)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.5 (2.7)</td>
<td>24.5 (3.3)</td>
<td>24.7 (2.7)</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>86.8 (7.4)</td>
<td>85.3 (9.3)</td>
<td>85.9 (8.0)</td>
</tr>
</tbody>
</table>

Gr.Suppl.0 = no vitamin D₃–fortified yogurt consumption; recommendation to maintain dietary habits during 24 weeks. Gr.Suppl.5 = 1 vitamin D₃–fortified yogurt/d during 16 weeks followed by 8 weeks without product. Gr.Suppl.10 = 2 vitamin D₃–fortified yogurts/d during 16 weeks followed by 8 weeks without product. BMI = body mass index.

*Values are means (SD). There was no statistically significant difference (overall analysis of variance) between the 3 groups for any of the 5 characteristics.

Discontinuation (WK24) is presented in Table 2. The baseline level was quite similar (about 36 nmol/L) among the 3 randomized groups and was within the insufficiency range in conformance with one prespecified inclusion criterion. From BSL to WK8, there was a dose-related increase in s25OHD of 12.3 and 18.4 nmol/L in Gr.Suppl.5 and Gr.Suppl.10, respectively (Table 2 and Figure 1A). From BSL to WK16, differences in changes were highly significant between Gr.Suppl.5 and Gr.Suppl.0 (18.3 vs 7.7 nmol/L, t test: p = 0.0007) and between Gr.Suppl.10 and Gr.Suppl.0 (23.5 vs 7.7 nmol/L, t test: p = 0.000001). The corresponding difference in change between Gr.Suppl.5 and Gr.Suppl.10 was not statistically significant (18.3 vs 23.5 nmol/L, y test: p = 0.116).

After 8 weeks of yogurt consumption, serum 25OHD was increased to 89.1% (Gr.Suppl.5) and to 94.1% (Gr.Suppl.10) of the level measured after 16 weeks (Table 2). In the control group (Gr.Suppl.0), during the first 8 weeks, s25OHD remained stable: 36.4 at BSL and 36.6 nmol/L at WK8 (Table 2, Figure 1A). Thereafter it rose significantly to reach a level of 44.1 nmol/L at week 16 (Table 2, Figure 1A). After discontinuation of the intervention, from WK16 to WK24, s25OHD was virtually maintained in both groups (Figure 1B). The rate constant was calculated based on the increase in s25OHD from BSL to WK16.

### s25OHD response in relation to baseline level

Based on screening s25OHD levels, the participants were segregated into low stratum (LowStr from 25 to 50 nmol/L, n = 53) and high stratum (HighStr ≥ 50 to 75 nmol/L, n = 80). The evolution from BSL to WK24 of the absolute s25OHD values indicates a dose effect of Vitamin D₃–fortified yogurts in both LowStr and HighStr subgroups (Table 3). The changes from baseline to 8 weeks were as follows: for Gr.Suppl.5: 17.4 nmol/L LowStr vs 8.4 nmol/L HighStr (Figure 2); for Gr.Suppl.10: 27.1 nmol/L LowStr vs 13.5 nmol/L HighStr; for Gr.Suppl.0: 3.0 nmol/L LowStr vs −1.6 nmol/mL HighStr (Figure 2). Thus, the absolute increase in s25OHD after 8 weeks was greater in LowStr than in HighStr regardless of the daily vitamin D₃ amounts consumed (5 or 10 μg).

### s25OHD rate constant

The rate constant was calculated based on the increase in s25OHD per microgram of Vitamin D₃ consumed from BSL to
parallel time-controlled group illustrate the influence of enrollment time (as designated by the term seasonality) in the absence of any VitD3 supplementation. In Gr.Suppl.0, the increase in s25OHD from BSL to WK16 was +1.1 nmol/L in the early group and +14.1 nmol/L in the late group. In Gr. Suppl.5, the corresponding increase was +15.2 nmol/L in the early group and +21.4 nmol/L in the late group. In Gr. Suppl.10, it was +12.5 nmol/L in the early group and +24.5 nmol/L in the late group (Table 4 and Figure 4). The contribution of the seasonality effect to the changes measured in the 2 groups consuming VitD3-fortified yogurts from BSL to WK16 may be roughly estimated by subtracting the corresponding change assessed in Gr.Suppl.0 (Table 4 and Figure 4).

Evolution of anthropometric, cardiovascular variables, and serum calcium

Over the 16 weeks of intervention, no significant change was observed in body weight, BMI, and waist circumference. Measurements of diastolic/systolic blood pressure remained stable in the 3 groups from BSL to WK8 and WK16. Likewise, serum calcium was not modified by the intervention.

Adherence to consumption of vitamin D3-supplemented yogurts

The degree of adherence as assessed by the number of yogurt pot lids returned by the participants at each visit compared to the number of theoretical number of yogurts distributed remained quite stable from WK4 to WK16, varying from 93% to 100% and from 82% to 100% in Gr.Suppl.5 and Gr.Suppl.10, respectively (p = 0.084).

Acceptability

The tested products, whether consumed at a daily rate of 1 or 2 fortified yogurts, were well accepted in terms of taste, with no negative feeling concerning the dairy product size, a factor that may reduce appetite and thereby limit food intake.

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></td>
<td>18</td>
<td>27</td>
<td>19</td>
</tr>
<tr>
<td>BSL</td>
<td>22.0 (7.4)</td>
<td>46.0 (12.1)</td>
<td>26.8 (10.4)</td>
</tr>
<tr>
<td>WK4</td>
<td>19.2 (8.8)</td>
<td>40.1 (12.0)</td>
<td>35.3 (9.4)</td>
</tr>
<tr>
<td>WK8</td>
<td>24.9 (10.5)</td>
<td>44.4 (14.9)</td>
<td>44.2 (11.1)</td>
</tr>
<tr>
<td>WK12</td>
<td>27.7 (13.0)</td>
<td>46.1 (16.8)</td>
<td>45.3 (12.3)</td>
</tr>
<tr>
<td>WK16</td>
<td>33.8 (16.2)</td>
<td>51.0 (15.8)</td>
<td>50.2 (13.3)</td>
</tr>
<tr>
<td>WK24</td>
<td>37.9 (14.1)</td>
<td>57.2 (17.7)</td>
<td>46.0 (13.5)</td>
</tr>
</tbody>
</table>

25OHD = 25-hydroxyvitamin D, Gr.Suppl.0 = no vitamin D3-fortified yogurt consumption; recommendation to maintain dietary habits during 24 weeks. Gr.Suppl.1 = vitamin D3-fortified yogurt/d during 16 weeks followed by 8 weeks without product, Gr.Suppl.10 = 2 vitamin D3-fortified yogurts/d during 16 weeks followed by 8 weeks without product. BSL = baseline visit, WK4/WK24 = number of weeks elapsed from the baseline visit, WK16 = end of the intervention for both Gr.Suppl.5 and Gr.Suppl.10. Gr.Suppl.10 = 8 weeks after discontinuation of the intervention for both Gr.Suppl.5 and Gr.Suppl.10.

*Serum 25OHD values are means (SD) in nanomoles per liter. The participants were distributed as low or high according to their serum 25OHD screening values; that is, before the BSL visit. Low stratum range: 25 to 50 nmol/L, high stratum range: ≥50 to 75 nmol/L. Probability levels for group-by-time interaction (repeated measures analysis of variance) for the evolution of serum 25OHD from BSL to WK16 were as follows: low stratum: p = 0.0001 and p = 0.0012 for the difference between Gr.Suppl.10 or Gr.Suppl.5 and Gr.Suppl.0, respectively, and p = 0.0346 for the difference between Gr.Suppl.10 and Gr.Suppl.5.
Discussion

Main results

This article highlights the relative importance of 3 interdependent determinants of the response to VitD3-fortified foods documented in one single randomized clinical trial carried out in menopausal women. It provides information on the impact of VitD3-fortified yogurt taken at 2 dose levels compared to a time-controlled group and on the quantitative influence of the baseline vitamin D status. It emphasizes the distinct impact of 2 amounts of VitD3 consumed orally in relation to the period of the year during which the intervention was conducted.

Relation to supplemental doses

According to a 2011 report from the Institute of Medicine, a s25OHD level of ≥50 nmol/L meets the needs, primarily related to bone health outcomes [4], of at least 97.5% of the population. In our study, the baseline s25OHD value of approximately 36 nmol/L (Figures 1A and 1B, Table 1) fell within the insufficiency range. Following consumption of the VitD3-fortified yogurts, s25OHD crossed the sufficiency threshold of 50 nmol/L earlier with a dose of 10 μg/d (54.3 nmol/L after 8 weeks) and later with a dose of 5 μg/d (54.8 nmol/L after 16 weeks; Table 1). Thus, the s25OHD kinetic response differentiates the 2 tested amounts of VitD3 better than the absolute level attained by the
end of the intervention (59.4 vs 54.8 nmol/L with 10 vs 5 μg/d after 16 weeks of intervention, \( p = 0.198 \); Table 2).

**Relation to initial s25OHD level**

Previous reports suggested an inverse correlation between baseline vitamin D status and an increase in s25OHD in response to either pharmaceutical supplement or food fortification [5,29,30]. The design of our randomized controlled study was prespecified to test this possible relationship. Our results clearly establish that the lower the baseline vitamin D status, the higher the absolute increment in s25OHD (Figure 2). The reasons for this inverse relationship remain conjectural. Among possible mechanisms are (1) the vitamin D status could influence the distribution of s25OHD; (2) the hepatic hydroxylation rate of the cholecalciferol molecule in position 25 could be inversely related to its product; (3) the affinity of the vitamin D binding protein(s) could vary according to vitamin D status; (4) the activity/induction of catabolic vitamin D 24-hydroxylase enzyme may be reduced in response to a prolonged decrease in the level of serum 25OHD [31–33].

**Rate constant of s25OHD 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 a supplement or fortified foods and improvement in vitamin D status. This value corresponds to an efficiency estimate of the supplemental VitD₃ consumed [18]. In a recent systematic review, the rate constant of change in s25OHD expressed as nanomoles per liter per microgram of additional vitamin D was calculated from 18 randomized controlled trials [5]. The mean rate constant close to 2.0 nmol/L per μg of vitamin D [5] was in agreement with an analysis of 41 studies with an average rate constant of 2.1 [34]. We observed an important effect of baseline s25OHD on the response to VitD₃-fortified dairy. It was more than twice as high in the participants randomized as LowStr (25 to 50 nmol/L) compared to HighStr (> 50 to 75 nmol/L; Figure 3).

**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 s25OHD. Our study ran from January to October and allows one to identify the relative importance of these 2 factors. Thus, the increase in response to VitD₃-fortified yogurts would be substantially overestimated if one did not take into account the evolution of s25OHD as assessed in our study by monitoring a parallel time-control group. The difference between the control and the 2 supplemental groups decreased from the onset of the intervention to the end (Table 1 and Figures 1A and 1B). Most likely, this attenuation of the fortified food effect results from the increased cutaneous production of VitD₃. In the control group, s25OHD significantly increased from 36.4 to 44.1 nmol/L (+21.2%) from BSL (mean sampling time mid-February) to WK16 (mean sampling time early June). It increased further to 49.5 nmol/L 8 weeks later (WK24; mean sampling time early August). 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 Institute of Medicine report [4]. This evolution is in keeping with the influence of seasonality on vitamin D status, as studied in some European countries and the United States [11–14,35]. The seasonality effect from 36.4 to 49.5 nmol/L in the control group (Table 1) approximately equals the consumption during 8 weeks of fortified dairy providing 5 μg/d of supplemental VitD₃, which increased the s25OHD level from 36.5 to 48.8 nmol/L (Table 1). 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 VitD₃ (Table 1). These results corroborate the utility of consuming supplemental vitamin D during the winter season.

**Food fortification for preventing vitamin D insufficiency in the general population**

All enrolled subjects remained compliant with the prescribed fortified dairy products, whether consumed as 1 or 2 servings per day during 16 weeks. This high adherence should reflect the good acceptability of supplemental VitD₃ as provided through the consumption of fortified dairy products (for a review, see Whiting et al. [36]).

In terms of public health programs aimed at preventing vitamin D insufficiency, adherence and acceptability are important criteria for achieving a beneficial effect of long-term supplementation. Relatively low vitamin D doses regularly consumed with usual foods offer some advantage over pharmaceutical pills that are either taken daily in small doses but with the risk of low compliance due to medication-related side effects [37] or, alternatively, taken in large amounts at monthly or yearly intervals with the nonnegligible risk of the occurrence of adverse events [38].

The results of our randomized trial in healthy menopausal women pertain to the prevention of insufficient vitamin D status in the general population. Quantitatively, the presented evolution of s25OHD can be interpreted in relation to the 2011 Institute of Medicine report that established that a s25OHD level of at least 50 nmol/L meets the skeletal health requirements for vitamin D of ≥97.5% of the general population [4]. Whether the benefits of vitamin D supplementation would only become manifest when s25OHD reaches a level of at least 75 nmol/L is controversial [3,39]. Furthermore, it remains uncertain that such a high level might be required for the adequate management of disease-related conditions such as cardiovascular disease, site-specific cancers, diabetes, chronic kidney disease, hepatic failure, malabsorption syndromes, and obesity [4,39]. In these pathologic conditions, dairy and/or non-dairy food fortification with vitamin D [40] may not be enough to achieve a s25OHD level of 75 nmol/L and above, particularly when ultraviolet B radiation is limited, as recommended to the participants in our trial. To achieve such high levels, the use of vitamin D pharmaceutical supplements in amounts close to the estimated tolerable upper intake level, set at 100 μg/day [4], appears appropriate.

Several epidemiological studies have shown an inverse association between s25OHD and age-adjusted mortality [41–43]. This association between vitamin D status and mortality appears to be stronger with cardiovascular disease (CVD) than non-CVD pathologies [41]. A doubling of s25OHD between 40 and 90 nmol/L was associated with a 20% lower vascular mortality [44].

In 2 recent reports on a well-conducted randomized placebo-controlled trial entitled BEST-D trial (Biochemical Efficacy and Safety Trial of Vitamin D), the effects of daily supplementation of 2000 IU (50 μg) and 4000 IU (100 μg) for 1 year were assessed in about 300 community-dwelling people aged ≥65 years in Oxfordshire [45,46]. s25OHD increased from a mean baseline level of 50 nmol/L to 102 and 137 nmol/L in those allocated daily VitD₃ doses of 50 and 100 μg, respectively [46], whereas it barely changed in the placebo group. The authors concluded that 100 μg of VitD₃ may be required to achieve a lowest risk of CVD and other diseases such as certain types of cancer [46]. Of note, supplementation with these high doses of VitD₃ had no detectable effects on cardiovascular risk factors, including blood pressure, arterial stiffness, circulating lipids, and markers of inflammation [46]. Nevertheless, this randomized controlled trial cannot exclude benefits of CVD prevention beyond 1 year of treatment with high doses of VitD₃.

**Strengths and weaknesses**

This study has a number of strengths:

1. It provides information from a randomized controlled trial on the interaction of 3 important determinants of the vitamin D status prospectively studied in 133 menopausal women who remained adherent over the 24 weeks of the investigation.
2. The kinetic analysis of the initial 8-week increment in s25OHD in response to the consumption of VitD₃-fortified dairy clearly differentiates the 2 daily doses tested, namely, 5 vs 10 μg.
3. The study also shows the attenuation of the difference between the 2 doses of supplemental VitD₃ during the 16 weeks of intervention.
4. It furthermore unequivocally documents the inverse relationship between baseline vitamin D status and the response to VitD₃-fortified yogurt.
5. It demonstrates the marked interaction between the effect of VitD₃ supplementation and the season-dependent onset of the intervention.
6. The study highlights the importance of VitD₃ supplementation during the winter season, even in amounts as low as 5 μg/d.
The marked in Suppl.10, respectively. The study unequivocally demonstrates seasonality.

4. The cohort was exclusively white and recruited in one single French region. Therefore, the results may not be the same for other ethnic groups or among populations with different dietary and lifestyle habits and/or living in regions at different latitudes and/or altitudes.

Conclusions
This clinical trial in menopausal women provides data on 3 interdependent determinants of vitamin D status. It documents a dose-response of vitamin D3-fortified yogurt on the evolution of s25OHD during 4 months and the 2 months following discontinuation of the intervention. At the end of the intervention, the percentage of subjects with s25OHD ≥ 50 nmol/L was about 38, 55, and 64% in Gr.Suppl.0, Gr.Suppl.5, and Gr. Suppl.10, respectively. The study unequivocally demonstrates the marked influence of the initial vitamin D status on the absolute increase in s25OHD: the lower the baseline status, the higher the response to a given dose of supplemental VitD3. The trial was conducted from January to October of the same year, which highlighted the important influence of season on the magnitude of the s25OHD response to 2 doses of VitD3-fortified yogurt compared to the maintenance of dietary habits in a time-controlled group. Thus, this randomized controlled trial quantitatively documents the interaction of 3 key determinants of vitamin D status: supplemental doses, initial status, and seasonality.

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Disclosure
J.P.B. is clinical consultant for General Mills-Yoplait; J.P.B. has no financial interest in the outcome of the reported clinical trial. F.D.-P. and B.R. are scientific collaborators of General Mills-Yoplait; E.R. is a former scientific collaborator of Yoplait France; and S.W. is scientific consultant for General Mills-Yoplait; S.W. has no financial interest in the outcome of the reported clinical trial.

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