Vitamin D status in kidney transplant patients: need for intensified routine supplementation

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ABSTRACT

Background: A high prevalence of vitamin D insufficiency has been found in the general population and in patients with chronic kidney disease.

Objective: The aim was to examine vitamin D status and determinants and metabolic correlates of serum 25-hydroxyvitamin D in a population of adult Danish kidney transplant patients.

Design: This was a cross-sectional study of 173 adult kidney transplant patients with a mean (±SD) age of 53.4 ± 11.7 y and a median graft age of 7.4 y (interquartile range: 3.3–12.7 y). Serum concentrations of intact parathyroid hormone (S-PTH), 25-hydroxyvitamin D [S-25(OH)D], and 1,25-dihydroxyvitamin D [S-1,25(OH)2D] were measured. Dietary and supplementary intake of vitamin D, avoidance of solar ultraviolet B exposure, and selected lifestyle factors were assessed in a subgroup (n = 97).

Results: Fifty-one percent of the patients had vitamin D insufficiency [S-25(OH)D 40–75 nmol/L], and an additional 29% had moderate-to-severe vitamin D deficiency [S-25(OH)D ≤ 39 nmol/L]. In multiple regression analysis, sun avoidance (negative association) and vitamin D supplementation (positive association) were independent determinants of S-25(OH)D concentrations. Low S-25(OH)D concentrations were associated with 1) increased S-PTH concentrations (P = 0.0002), independently of S-1,25(OH)2D concentrations, and 2) decreased S-1,25(OH)2D concentrations (P = 0.002), independently of graft function.

Conclusions: Hypovitaminosis D is common among Danish kidney transplant patients and is associated with reduced concentrations of S-1,25(OH)2D and increased S-PTH concentrations. Sun avoidance and vitamin D supplementation are important determinants of vitamin D status. The observed hypovitaminosis D might be corrected by intensified routine vitamin D supplementation as opposed to the current supplementation practice. Am J Clin Nutr 2008;87:431–7.

KEY WORDS Kidney transplantation, hypovitaminosis D, prevalence, sun exposure, vitamin D intake, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, parathyroid hormone

INTRODUCTION

The serum concentration of 25-hydroxyvitamin D [S-25(OH)D] is considered an accurate integrative measure of vitamin D status because it reflects both an individual’s intake and the cutaneous production of vitamin D (1–3). Natural dietary sources of vitamin D are limited to a few foods of importance: mainly fish and to a lesser degree meat, eggs, milk, and dairy products (4). Thus, exposure to solar ultraviolet B (UVB) is considered crucial to satisfying requirements for vitamin D (5). The risk of an insufficient cutaneous vitamin D synthesis is increased in populations living in countries at high latitudes with limited sun irradiation, such as the Nordic countries. This risk is further increased among the elderly, certain ethnic minorities, and the chronically ill (6–9). Accordingly, a high prevalence of vitamin D insufficiency has been found in the general population (eg, in Denmark) and in patients with chronic kidney disease (CKD) (10–15).

Kidney transplant patients are advised to avoid direct sun exposure because their immunosuppressive therapy is associated with a higher risk of skin carcinomas (16). Consequently, they are at particular risk of hypovitaminosis D. The main purpose of the present study was to examine the prevalence of hypovitaminosis D, determinants of vitamin D status, and interrelations between vitamin D status, 1,25-dihydroxyvitamin D [1,25(OH)2D], and intact parathyroid hormone (PTH) in a well-defined population of adult Danish kidney transplant patients.

SUBJECTS AND METHODS

Subjects

This cross-sectional study was performed between 1 December 2005 and 1 April 2006, ie, a period when S-25(OH)D is expected to be at its annual nadir. All 242 adult (>18 y of age) kidney transplant patients followed in the outpatient nephrology clinic at Copenhagen University Hospital Herlev were invited to participate. Exclusion criteria were acute illness, life-threatening competitive illness, mental disorders, and need for dialyses. Within a predefined time limit of 10 d, exactly 100 patients had given their written consent to participate in all parts of the study (including a blood sample, dual-energy X-ray absorptiometry, muscle tests, and an interviewer-administered questionnaire). Three of the 100 patients were subsequently excluded because of...
their need for dialyses. An additional 76 of the 242 invited patients gave their written consent to participate after ≥10 d. Because of logistics, their participation had to be limited to blood sampling. The present study is based on blood samples from all participants (97 + 76) and questionnaire data from the 97 patients. The study was approved by the Regional Ethics Committee of Copenhagen County (j.no.KA 05078).

Biochemical measurements

Nonfasting blood samples were drawn between 0900 and 1300. Laboratory variables included measurements of serum intact parathyroid hormone (S-PTH), S-25(OH)D, and 1,25(OH)₂D₃ [S-1,25(OH)₂D₃], creatinine, urea, albumin, phosphate, and ionized calcium.

S-25(OH)D (D₂ and D₃) concentrations (nmol/L) were assessed with a competitive radioimmunoassay (DiaSorin, Minneapolis, MN) in the clinical biochemical laboratory at Copenhagen University Hospital Herlev. The lower detection limit given by the manufacturer was 4.0 nmol/L, and the interassay precision (CV) in the laboratory was 11.4%. We defined vitamin D insufficiency and deficiency according to the recently published Kidney Disease Outcome Quality Initiative (K/DOQI) guidelines from the National Kidney Foundation (17). Thus, vitamin D status was considered adequate when S-25(OH)D concentrations were ≥75 nmol/L (≥30 ng/mL). Concentrations between 40 and 75 nmol/L (16–30 ng/mL) represent vitamin D insufficiency. Vitamin D deficiency was defined as S-25(OH)D concentrations ≤39 nmol/L (≤15 ng/mL) and was further subdivided into moderate (12–39 nmol/L, or 5–15 ng/mL) and severe (<12 nmol/L, or <5 ng/mL) vitamin D deficiency. In this article hypovitaminosis D was defined as an S-25(OH)D concentration <75 nmol/L.

S-1,25(OH)₂D₃ (D₂ and D₃) concentrations (pmol/L) were assessed with a competitive radioimmunoassay (Immuno Diagnostic Systems, Bolden, United Kingdom) in the clinical biochemical laboratory at Aarhus University Hospital. According to the manufacturer, the lower and upper detection limits were 5 and 550 pmol/L, respectively, and the interassay CV was 6.8%.

S-PTH concentrations (ng/L) were assessed by ADVIA Centaur Intact PTH (Bayer HealthCare, Tarrytown, NY) in the clinical biochemical laboratory at Copenhagen University Hospital Herlev. The laboratory interassay CV was 8.4%.

The serum concentrations of creatinine (reference interval: 40–110 μmol/L), urea (reference interval: 2.5–7.5 mmol/L), albumin (reference interval: 550–770 μmol/L), phosphate (reference interval: 0.8–1.5 mmol/L), and ionized calcium (reference interval: 1.15–1.35 mmol/L) were measured according to standard procedures in the clinical biochemical laboratory at Copenhagen University Hospital Herlev. Laboratory interassay CVs were 2.1% for creatinine, 2.1% for urea, 2.1% for albumin, 2.3% for ionized calcium, and 1.4% for phosphate.

Calculation of glomerular filtration rate

Kidney function was assessed from the estimated glomerular filtration rate (eGFR) according to the equation from the Modification of Diet in Renal Disease Study (18) as recommended by K/DOQI guidelines (19): 170 × serum creatinine (mg/dL)⁻⁰.₉⁹⁹ × age (y)⁻₀.₁₇⁶ × 0.762 (if patient is female) × serum urea (mg/dL)⁻₁.₀₁₇ × serum albumin (g/dL)⁻₀.₃₁₈. The estimated GFR (eGFR) is expressed in mL · min⁻¹ · 1.73 m⁻².

Interview

On the day of blood sampling, patients (n = 97) answered an interviewer-administered questionnaire. The questionnaire included questions concerning the patients’ smoking habits, avoidance of sun exposure, physical activity level, self-reported use of vitamin D and calcium supplements, and a standardized food-frequency questionnaire (FFQ) that ascertained the dietary intake of vitamin D and calcium.

Dietary intake of vitamin D and calcium

The patients’ dietary intake of vitamin D and calcium within the past month was assessed with a FFQ with food items contributing to 95% of the vitamin D intake and 75% of the calcium intake determined from the most recent Danish dietary survey “Dietary Habits in Denmark, 2000–2002” (20) as described previously (10). The FFQ had 9 possible frequencies (ranging from “less than one time per month” to “4–5 times per day or more”). The FFQ, standardized recipes, portion sizes, and water and fat contents were kindly provided by the Danish Institute for Food and Veterinary Research (now the National Food Institute, DTU). Dietary data were calculated by using the software program Dankost 3000a (Dansk Catering Center, Herlev, Denmark).

Vitamin D and calcium supplements

The patients were asked to bring self-selected dietary supplements (eg, multivitamins and cod-liver oil) containing calcium or vitamin D (ergo- and cholecalciferol) with them to the interview. Daily doses and types of supplement were recorded. Prescribed amounts vitamin D and calcium derived from the patients’ medical records were recorded. The only active vitamin D analogue used was alphacalcidiol (Onealpha; Leo Pharma A/S, Ballerup, Denmark). Alphacalcidiol could only be obtained with a prescription.

Sun exposure

Patients were categorized into 1 of 3 groups (sun avoidance, partial sun avoidance, or no sun avoidance) according to a score obtained from the following questions concerning avoidance of sun exposure: “Do you stay directly in the sun in the summer period between 11.00 a.m. and 3.00 p.m.?” “Do you use sun protection on all parts of your body in the sun?” “When in the sun, do you always wear: a hat or a cap?, long sleeves? and/or long trousers?”

Other variables

The patients were asked about their smoking status (non-smoker, ex-smoker, or current smoker). Weight and height were recorded without shoes. The body mass index (BMI; in kg/m²) was calculated. The age of the patients is the age on the date of blood sampling. Additional variables were assessed (eg, muscle function and bone mineral density) and will be presented separately in other articles.

Statistical analysis

Analyses included standard descriptive statistics. Normally distributed variables are presented as means ± SDs, whereas nonnormally distributed variables are presented as medians and interquartile ranges (IQRs). Group differences were tested by using an unpaired t test or Mann-Whitney U test as appropriate.
The significance level was chosen as $P < 0.05$. The analyses were performed with the software program SAS for WINDOWS (version 9.1; SAS Institute Inc, Cary, NC).

A backward multiple linear regression analysis was performed to describe the relation between square root–transformed S-25(OH)D and possible explanatory variables. The following categorical variables were included: smoking status (nonsmoker, ex-smoker, or current smoker), diabetes (yes or no), and alphacalcidol supplementation (yes or no).

The following continuous numerical variables were included: age (y), BMI (kg/m²), dietary vitamin D intake ($\mu$g/d), S-PTH (ng/L), eGFR (mL·min⁻¹·1.73 m⁻²), vitamin D supplementation ($\mu$g/d), and S-albumin (mg/L).

A second backward multiple linear regression analysis was performed to describe the relation between square root–transformed S-1,25(OH)₂D as the dependent variable and S-25(OH)D, adjusted for other possible explanatory variables. The following categorical variables were included: smoking (nonsmoker, ex-smoker, or current smoker), alphacalcidol supplementation (yes or no), and sex (female or male). The following continuous numerical variables were included: serum ionized calcium (mmol/L), vitamin D supplementation ($\mu$g/d), age (y), serum cholesterol (mmol/L), serum PTH (ng/L), dietary vitamin D intake ($\mu$g/d), eGFR (mL·min⁻¹·1.73 m⁻²), and serum phosphate (mmol/L).

A third backward multiple linear regression analysis was performed to describe the relation between (logarithmic transformed) S-PTH as the dependent variable and S-25(OH)D and S-1,25(OH)₂D, adjusted for other possible explanatory variables. The following categorical variables were included: sex (female or male) and diabetes (yes or no). The following continuous numerical variables were included: total (from diet and supplements) calcium intake (mg/d), serum ionized calcium (mmol/L), serum albumin (mg/L), serum phosphate (mmol/L), age (y), and eGFR (mL·min⁻¹·1.73 m⁻²).

RESULTS

There were equal numbers of women ($n = 87$; mean ± SD age: 53.6 ± 11.8 y) and men ($n = 86$; mean ± SD age: 53.2 ± 11.6 y) in our study population ($n = 173$). The primary renal diagnoses were glomerulonephritis (27%), polycystic kidney disease (14%), diabetic nephropathy (14%), hypertensive nephropathy (8%), chronic interstitial nephritis (8%), others (9%), and unknown causes (20%). The large majority of the patients were ethnic Danes; 9% were dark-skinned.

Median kidney graft age was 7.4 (IQR: 3.3–12.7) y, median eGFR was 38.9 (IQR: 27.9–51.2) mL·min⁻¹·1.73 m⁻², median serum creatinine was 154 (IQR: 117–200) $\mu$mol/L, median serum urea was 10.8 (IQR: 7.9–15.2) mmol/L, and median serum PTH was 97 (IQR: 63–156) ng/L. The following 3 blood measurements were within normal ranges: serum albumin (mean ± SD: 624 ± 54 $\mu$mol/L), serum ionized calcium (median: 1.25 mmol/L; IQR: 1.22–1.31 mmol/L), and serum phosphate (median: 1.05 mmol/L; IQR: 0.87–1.20 mmol/L).

In the subgroup that was interviewed and had anthropometric measurements made ($n = 97$), the average woman was aged 55.1 ± 12.3 y (mean ± SD) and had a BMI of 24.4 (IQR: 22.4–27.5). The average man was aged 55.9 ± 12.1 y and had a BMI of 27.3 ± 5.0.

FIGURE 1. Prevalence of severe [serum 25-hydroxyvitamin D (S-25(OH)D) < 12 nmol/L] and moderate [S-25(OH)D: 12–39 nmol/L] vitamin D deficiency, vitamin D insufficiency [S-25(OH)D: 40–75 nmol/L], and vitamin D sufficiency [S-25(OH)D ≥ 75 nmol/L] according to Kidney Disease-Outcome Quality Initiative guidelines in the present study population of kidney transplant patients ($n = 173$; chi-square test, $P = 0.03$).

Vitamin D status and 1,25(OH)₂D concentrations ($n = 173$)

The vitamin D status of women and men is shown in Figure 1. Only 26% of the women and 12% of the men had desirable S-25(OH)D concentrations (≥75 nmol/L). Among women and men, respectively, 48% and 55% had vitamin D insufficiency (40–75 nmol/L), whereas an additional 26% and 33% had moderate-to-severe vitamin D deficiency (≤39 nmol/L). The median S-25(OH)D concentration was significantly higher ($P < 0.05$) in women (median: 54.0 nmol/L; IQR: 39–78 nmol/L) than in men (median: 45.5 nmol/L; IQR: 30–67 nmol/L).

The distribution of S-1,25(OH)₂D is shown in Figure 2. Most patients had S-1,25(OH)₂D concentrations within the normal range (60–180 pmol/L). Fourteen percent of the women and 15% of the men had S-1,25(OH)₂D concentrations <60 pmol/L.

Dietary and supplementary intake of vitamin D and sun exposure ($n = 97$)

The median dietary intake of vitamin D was 3.2 (IQR: 1.9–4.8) $\mu$g/d in women ($n = 54$) and 3.1 (IQR 1.5–4.8) $\mu$g/d in men ($n = 43$). These values are similar to the average intake of 3.3 $\mu$g/d in the general Danish adult population (20). Sixty-nine percent of the women and 51% of the men took vitamin D supplements. In about one-third of these cases, the vitamin D supplements had been prescribed by a physician. Accordingly, two-thirds took vitamin D supplements on their own initiative. The median daily dose of vitamin D was 10 $\mu$g in women and 5 $\mu$g in men. Alphacalcidol was prescribed to 20% of the women and to 16% of the men. The average S-25(OH)D concentration according to vitamin D or alphacalcidol supplementation is presented in Table 1.

Seventeen percent of the women and 14% of the men avoided sun exposure, 72% of the women and 65% of the men only avoided sun exposure to some extent, whereas 11% of the women...
Determinants of S-25(OH)D

The results (estimates, SEs, and P values) of the multiple linear regression analysis with square root–transformed S-25(OH)D as the dependent variable are presented in Table 2. A significantly positive association was found with vitamin D supplementation, age, and S-albumin and a significantly negative association was found with current smoking, sun avoidance, and BMI. Approximately 54% of the total variation in the S-25(OH)D concentrations could be explained by the included independent variables. The association between vitamin D and S-25(OH)D according to sun avoidance is shown in Figure 3. In the average patient who did not avoid sun exposure, a daily intake of 12 μg vitamin D is needed to meet the recommended S-25(OH)D concentration of ≥75 nmol/L. Correspondingly, a daily intake of 22 μg vitamin D is needed by the average patient who avoids sun exposure.

TABLE 1

Serum 25-hydroxyvitamin D concentrations in users and nonusers of vitamin D supplements

<table>
<thead>
<tr>
<th>Vitamin D (ergo and cholecalciferol)</th>
<th>User</th>
<th>Nonuser</th>
<th>All</th>
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<tr>
<td></td>
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<tr>
<td>nmol/L</td>
<td></td>
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<tr>
<td>Alphacalcidol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonuser</td>
<td>68 ± 3.4 [51]</td>
<td>43 ± 3.6 [28]</td>
<td>60 ± 2.9 [79]</td>
</tr>
<tr>
<td>All</td>
<td>66 ± 3.2 [59]</td>
<td>42 ± 3.2 [38]</td>
<td>57 ± 2.6 [97]</td>
</tr>
</tbody>
</table>

All values are x ± SEM; n in brackets. Data only available for the interviewed subgroup of patients (n = 97). Group differences were tested by using an unpaired t test. Significant difference between superscript letters a and b (P < 0.05), c and d (P < 0.0001), and e and f (P < 0.0001).

Determinants of serum 1,25(OH)2D and serum PTH

A positive association was found between S-25(OH)D and square root–transformed S-1,25(OH)2D (P = 0.002). This association was independent of the other variables in the multiple regression analysis, including eGFR. Additionally, an inverse association was found between S-25(OH)D and (logarithmic transformed) S-PTH (P = 0.0002), whereas the inverse association between S-1,25(OH)2D and logarithmic-transformed S-PTH was not significant (P = 0.069).

DISCUSSION

Fifty-one percent of the study population had vitamin D insufficiency as defined by K/DOQI guidelines. An additional 30% of the patients had definite vitamin D deficiency, meaning that the prevalence of hypovitaminosis D totalled 81%. Our study was conducted during a winter period with no cutaneous production of vitamin D. The values may be somewhat smaller during the summer, but, nonetheless, our findings correspond with earlier reports of kidney transplant patients (21–24) and confirm that their prevalence of hypovitaminosis D is unacceptably high.

In the general Danish population, previous studies found that 33–40% had vitamin D insufficiency [defined as S-25(OH)D < 50 nmol/L in healthy individuals] and an additional 7–10% had vitamin D deficiency [S-25(OH)D < 25 nmol/L in healthy individuals] during the winter (25). Using the threshold values defined for healthy individuals, 40% of our study population had vitamin D insufficiency and 14% vitamin D deficiency, which is only marginally higher than the values for the healthy Danish population. The problem of hypovitaminosis D is thus not uniquely different between kidney transplant patients and the general population of a Nordic country such as Denmark. However, this problem may be particularly worrisome in such patients for several reasons. First, severe hypovitaminosis D may cause the classic clinical signs of osteomalacia, such as myopathy, fatigue, muscle, and bone pain (26–30). Second, hypovitaminosis D may add to the higher risk of osteoporosis and fractures in kidney transplant patients (22, 31, 32). Third, vitamin D has several important nonclassic effects in the body. Besides possible anticarcinogenic properties, vitamin D seems to stimulate the innate immune system, and thus improve the body’s defense against infections, and to modify the function of T lymphocytes of the immune system (33, 34). According to recent reports,
hypovitaminosis D tends to augment T cell reactivity, which might be associated with an elevated risk of graft rejection (33). For all of these reasons, we find it highly essential that states of hypovitaminosis D are detected and corrected, especially in kidney transplant patients.

The average dietary intake of vitamin D in our study population was very close to the (low) dietary intake in the general population. This finding indicates that the marginally higher prevalence of hypovitaminosis D found in kidney transplant patients than in the general population was not due to unusual dietary habits.

As expected, S-25(OH)D concentrations were significantly lower in sun avoiders than in those who did not avoid the sun and in nonusers of supplements than in users of vitamin D supplements. Kidney transplant patients are recommended to avoid sun exposure because of their highly increased risk of skin cancer. This means that these patients are deprived of the most important source of vitamin D. Effective dietary vitamin D sources are very few and practically nonexistent. Therefore, vitamin D supplementation seems to be the only feasible means of improving and correcting vitamin D status in (kidney) transplant patients. According to our model (Figure 3), the average kidney transplant patient who avoids the sun needs a daily supplement of 22 µg vitamin D to reach the desired S-25(OH)D concentration of 75 nmol/L. To ensure S-25(OH)D concentrations ≥75 nmol/L in all kidney transplant patients, even higher doses of vitamin D need to be prescribed. We propose that a daily supplement of 30 µg vitamin D would satisfy the need in most patients. The difference in biological potency between ergo- and cholecalciferol necessitates routine monitoring of S-25(OH)D whatever kind of vitamin D supplement is actually chosen (35, 36).

There is an ongoing debate about the safety of vitamin D supplementation in CKD patients. The hepatic 25-hydroxylation of endogenously produced or exogenously obtained vitamin D is relatively fast and unregulated, and the biological half-life of S-25(OH)D is ≈2–3 wk, whereas the half-life of S-1,25(OH)2D is only 4–6 h. Vitamin D excess (hypervitaminosis D) may lead to hypercalcemia, hyperphosphatemia, and hypercalciuria. A recommended upper limit of 250 µg/d vitamin D was recently proposed for healthy adults (37). K/DOQI guidelines consider daily doses of 25–50 µg ergocalciferol to be safe and recommend the monitoring of serum calcium and phosphate as a precaution in supplemented patients (17).

Interestingly, patients prescribed alphacalcidol had significantly lower S-25(OH)D concentrations than did the remaining patients (Table 1). This difference could not be explained by lower dietary vitamin D intakes or less sun exposure (data not shown). Whatever the explanation might be, our finding suggests that hypovitaminosis D tends to be overlooked in patients treated with alphacalcidol. Alternatively, it could indicate that some physicians consider the S-25(OH)D concentration to be unimportant if alphacalcidol is prescribed. The latter standpoint is challenged by the fact that S-25(OH)D serves as a substrate for 1α-hydroxylases of several extrarenal tissues (38–40). Thus, inadequate serum concentrations of 25(OH)D may have a negative effect on the extrarenal, locally regulated synthesis of 1,25(OH)2D. Furthermore, 25(OH)D may play an important role in activating vitamin D receptors (VDRs) despite its lower VDR affinity than that of 1,25(OH)2D. Therefore, circulating 25(OH)D concentrations are ≈500–1000 times those of S-1,25(OH)2D concentrations (41). Our observation that S-25(OH)D was and S-1,25(OH)2D was not significantly associated with S-PTH supports that optimal S-25(OH)D concentrations are indeed
of importance, even in patients treated with alphacalcidol. Accordingly, we find that S-25(OH)D concentrations should be optimized irrespective of ongoing treatment with active vitamin D metabolites, unless vitamin D supplementation is contraindicated for other reasons. This opinion agrees with current K/DOQI guidelines on vitamin D therapy for CKD patients (stages 1–4) with hypovitaminosis D. The same guidelines also stress that alphacalcidol or calcitriol therapy only should be considered if the S-25(OH)D concentration is ≥75 nmol/L (17). Randomized intervention studies are clearly needed to determine whether such a strategy results in clinical benefits in relation to secondary hyperparathyroidism, renal osteodystrophy, and fracture rates in kidney transplant patients.

In conclusion, we found a high prevalence of hypovitaminosis D in Danish kidney transplant patients. Because of the necessity of sun avoidance in this patient group and the limited dietary sources of vitamin D, all kidney transplant patients should be prescribed vitamin D supplements. According to our calculations, a rather high daily dose of 22–30 μg vitamin D is needed to reach desirable S-25(OH)D concentrations. An increased awareness of the vitamin D status of kidney transplant patients and appropriate treatment of hypovitaminosis D may help improve these patients’ bone and muscle health and may be associated with additional beneficial effects.

We thank the National Food Institute for kindly providing the FFQ and other materials (eg, standardized recipes).

The authors’ responsibilities were as follows—PM: designed and supervised the study and contributed to the data interpretation and significant consultation; AG and BE: collected data, performed the statistical analysis, contributed to the data interpretation, and wrote the manuscript; AMF: collected data; and CM: contributed to the data interpretation. All authors participated in the revision of the manuscript and approved the final version of the manuscript. None of the authors had a personal or financial conflict of interest.

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