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Factors associated with serum 25-hydroxyvitamin D concentrations in older people in Europe: The EUREYE study

Running head: Serum 25OHD and related factors.

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Abstract

Background/Objective: We aimed to describe serum 25-hydroxyvitamin D (25OHD) concentrations in older Europeans and to investigate associations between 25OHD and lifestyle factors, including dietary intake and supplement use.

Subjects/Methods: Men and women aged ≥ 65 years were recruited from seven centres across north to south Europe. Serum 25OHD₂ and 25OHD₃ concentrations were measured by liquid chromatography tandem mass spectrometry (LC-MS/MS) in 4495 samples and total 25OHD (25OHD₂ + 25OHD₃) was adjusted for season of blood collection.

Results: The mean (25th, 75th quartile) of seasonally adjusted 25OHD was 46 (34, 65) nmol/L, with the highest concentration of 25OHD in Bergen [61 (49, 79) nmol/L], and the lowest in Paris [36 (24, 57) nmol/L]. Vitamin D deficiency (25-50 nmol/L) and vitamin D insufficiency (50-75 nmol/L) were found in 41% and 33% of the population, respectively. In multivariable analysis controlled for confounders, seasonally adjusted 25OHD concentrations were significantly ($p < 0.05$) lower in smokers and participants with self-reported diabetes and higher with increasing dietary vitamin D, and supplement use with fish liver oil, omega-3 and vitamin D. Additionally in further analysis excluding Bergen, 25OHD was associated with higher intakes of oily fish and increasing UVB exposure. We observed low concentrations of 25OHD in older people in Europe.

Conclusions: Our findings of the higher 25OHD concentrations in supplement users (omega-3 fish oil, fish liver oil, vitamin D) add to current recommendations to reduce vitamin D deficiency. We were unable to fully assess the role of dietary vitamin D as we lacked information on vitamin D fortified foods.

Introduction

Vitamin D deficiency (<50 nmol/L) is highly prevalent in older people (1–4). Serum 25-hydroxyvitamin D (25OHD) concentrations in Central Europe can vary from 32.5 to 72.5 nmol/L, with seasonal variability present (5). The main source of vitamin D is the endogenous production of vitamin D₃ from sunlight (6). However, seasonal and latitudinal effects in Europe can reduce the production of vitamin D₃ from the sun (7–10). The main sources of dietary vitamin D are oily fish and eggs (11,12), however, eggs despite being considered an important source of vitamin D, have a relatively low dietary vitamin D content. There is also evidence to suggest meat may be a vital source of dietary vitamin D (13). Social isolation and reduced mobility, both of which can lead to a decrease in the time spent outdoors, has been suggested as a reason for lower concentrations of total 25OHD (Total 25OHD= 25OHD₂ + 25OHD₃) in older individuals (14). Effective dietary strategies, including supplements, have been recommended by a number of research organisations and research groups for an older population group (15–17). 40-100 micrograms of supplement vitamin D has been suggested in addition to safe UVB exposure. This particular group have suggested vitamin D deficiency be defined as <50 nmol/L, 50-75 nmol/L as suboptimal and 75-125 nmol/L as optimal vitamin D concentration (18). It has also been suggested that target vitamin D concentrations and recommended vitamin D supplement values should be age-, body weight-, disease status- and ethnicity-dependent rather than focused on age alone. In addition, the recommendations should also depend on one's individual health outcome concerns, latitude of residence, dietary and cultural habits. This would ensure national/international guidelines would be more applicable to clinical practices (19).

There is a large amount of variability in the literature regarding 25OHD concentrations in different countries(20–23). The use of different assays and discrepancies over the definition of vitamin D deficiency makes the comparison of literature from various countries and in

different population groups difficult. A recent paper by Cashman and colleagues tried to address this issue by applying vitamin D standardization program (VDSP) protocols to existing 25OHD data from 55844 participants from representative studies for European children, teenagers, adults and older adults. The authors concluded that vitamin D deficiency (defined as <30 nmol/L) was highly prevalent in Europe, with 13% of individuals being deficient throughout the year. Deficiency throughout the winter months (November to March) was 18%, and 8% in the summer months (April-October)(21).

The European Eye (EUREYE) study was a cross-sectional study of older people from centres in seven countries spanning north to south Europe, originally set up to investigate risk factors for age-related macular degeneration. In previous analyses we reported the association between vitamin D deficiency and age-related macular degeneration (24) and total 25OHD concentrations and myopia (25). In the present analysis, we aimed to further describe 25OHD concentrations, including prevalence of vitamin D deficiency, and to investigate the association of key demographic, clinical and lifestyle factors, including dietary factors, with 25OHD concentrations.

Research materials and methods

The methods for the EUREYE study have been published in detail elsewhere (26). Briefly, participants were recruited during 2001 and 2002 by random sampling of the population aged 65 years and older in seven centres: Bergen, Norway; Tallinn, Estonia; Belfast, Northern Ireland; Paris, France; Verona, Italy; Thessaloniki, Greece; and Alicante, Spain. Over 11000 people were invited, of whom 5040 participated (45% response rate) (27). The response rate ranged from 35% (Verona) to 59% (Tallinn), in men (50%) compared to women (42%) and in the age group 65-74 (48%) compared to those 75 years and over (39%). Written informed consent was obtained from all study participants. Ethical approval was obtained for each centre from the relevant ethics committee and the research adhered to the tenets of the Declaration of Helsinki.

Participants were interviewed at the research clinic in each centre on a one to one basis by trained fieldworkers using a structured questionnaire. Information including age, smoking, alcohol use, previous cardiovascular events (angina, stroke, and heart attack) or history of diabetes mellitus, diet and sunlight exposure was obtained. Details of the diet (28) and sunlight questionnaires (29) have been described elsewhere and are summarised briefly here.

The UK European Prospective Investigation into Cancer and Nutrition (EPIC) study food frequency questionnaire (FFQ) (30) was used and modified in non-UK centres to include additional food items or local varieties of a food item. The intake of oily and white fish was asked about separately. Information on supplement use was recorded. Quantification of dietary intake of vitamin D was based on food sources alone, as the FFQ was not designed to facilitate the quantification of vitamin D intake from the diet via food fortification. Nutrient intake including vitamin D was estimated using the food-composition tables from the 5th edition of McCance and Widdowson's "The Composition of Foods" (31). Dietary vitamin D

was adjusted for total energy intake using the residual model (32). We used a sunlight questionnaire to ask about place of residence and time spent outdoors between the hours of 9 a.m. and 5 p.m. and between 11 a.m. and 3 p.m daily. Estimates of exposure for different wavelengths of light (UVA, UVB and blue light) were generated from questionnaire data along with estimates from published sources that take into account time of day, month, and latitudinal variations modified by cloud cover and terrain. In these analyses we used middle of the day estimates for UVB as this is the period when sun exposure is at its highest.

At the clinical exam, systolic and diastolic blood pressure, weight and demispan were measured and a non-fasting blood sample collected from each participant. Blood samples were separated within 4 hours of collection and serum stored at -20° C for up to 4 weeks before being transferred to a single laboratory (Queens University Belfast) for storage at -80° C.

The full procedure for the measurement of 25OHD₂/25OHD₃ has been published elsewhere (33). Briefly, serum samples were prepared using a liquid-liquid extraction technique. Working standards and quality control samples were prepared by diluting commercially available 25OHD₂ and 25OHD₃ bi-level serum controls (Chromsystems, Munchen, Germany) into horse serum (Sigma- Aldrich Co Ltd, Poole, UK). A d6-25OHD₃ internal standard was used in both standards and serum samples. Samples were analysed for 25OHD₂ and 25OHD₃ by liquid chromatography tandem mass spectrometry (LC-MS/MS) [Waters® Xevo TQ-S® & ACQUITY UPLC (Waters Corporation, Milford, MA, USA)]. The intra-assay precision ranged from 1.6-3.6% and 1.1-4.0% for 25OHD₂ and 25OHD₃, respectively. The inter-assay precision ranged from 1.2-5.7% and 1.2-3.0% for 25OHD₂ and 25OHD₃, respectively. These measurements took place on the stored samples some 8 to 10 years after initial collection.

Statistical analysis

Statistical analysis was carried out using STATA 14 (StataCorp, College Station, TX, USA.). Normal distribution of variables was tested using normality plots and, subsequently, dietary vitamin D and total 25OHD (25OHD₂ + 25OHD₃) were logarithmically transformed. Season was defined by the date of blood collection as winter (December, January, February), spring (March, April, May), summer (June, July, August) and autumn, (September, October, November). Season was accounted for using sine wave analysis using the month of blood collection. Analysis using 25OHD was seasonally adjusted where required. Serum 25OHD was defined as grossly deficient (<25 nmol/L), deficient (25-50 nmol/L) and insufficient (50.75 nmol/L) as per published guidelines (16).

Seasonally adjusted total 25OHD concentrations were compared between two categories using an independent samples t test. For more than two categories, ANOVA was used. Categorical variables were compared using chi-square analysis. Variables were entered into multivariate analysis if their p-value was less than 0.05 in the univariate analysis. Multiple linear regressions were used to determine factors which were independently associated with 25OHD by adding the variables into a general linear model. Beta coefficients and 95% confidence intervals (CI) in the multivariable analysis are reported.

Study design (7 centres) was accounted for by using the STATA survey command in the estimation of means, corresponding p values and 95% CI. Design-adjusted Pearson chi-square tests were used for comparing categorical variables with categorical variables and design-adjusted Wald tests were used for comparing continuous variables with categorical variables.

As 25OHD and dietary vitamin D were log-transformed, mean and 95% CI were presented as geometric mean and 25th-75th quartiles. Pearson correlation coefficients and the multiple

regression coefficients which used a logged variable as a dependent variable were back transformed.

Results

Blood samples were available for 4704 participants (93.4%), of who 191 had no information on date of blood collection. A further 18 participants with total 25OHD concentrations greater than 150 nmol/L (11 due to implausibly high 25OHD₂ values) were excluded, leaving a final sample of 4495 available for analysis.

Demographic and lifestyle characteristics of study participants are presented in Table 1. The mean age was similar across the seven centres (71 to 74 years). UVB exposure was lower in north European centres compared to southern European centres. Paris had the lowest UVB exposure and Thessaloniki the highest. The BMI of participants in the normal (18.5-25.0 kg/m²), overweight (>25.0 kg/m²), and obese (>30.0 kg/m²) categories varied across the centres. Prevalence of obesity ranged from 19.8% in participants from Belfast to 51.0% in Thessaloniki. Similarly, the prevalence of self-reported diabetes was highest in Thessaloniki (23.4%). Current smoking status varied among the centres, Paris had the lowest percentage of smokers (8.2%), with Thessaloniki and Bergen having twice as many (18.3 and 18.1%, respectively) compared to Paris. The use of fish liver oil or, omega-3 fish oil and vitamin D supplements varied across the centres: Bergen had the highest number of omega-3 supplement users (43.7%), and Belfast for fish liver oil (24.3%). The remaining centres had extremely low intakes of fish liver oil or omega-3 supplement use, for example, Alicante had no supplement users. Vitamin D supplement use was low in the seven study centres, with Bergen having the highest percentage of supplement users (3.9%), and Thessaloniki having no users of vitamin D supplements.

Figure 1 shows the comparison of 25OHD concentrations by season overall and by centre. Overall, participants whose blood were collected between December and May had significantly lower 25OHD concentrations compared to those collected between June and

November (41 and 51 nmol/L, respectively $p < 0.001$). Seasonal differences were observed between each centre ($p < 0.001$), with the exception of Bergen ($p = 0.55$).

Table 2 shows the seasonally adjusted geometric mean and 25th, 75th quartiles for 25OHD, prevalence of participants who were grossly deficient (< 25 nmol/L), deficient (25-50 nmol/L) and insufficient (50-75 nmol/L) in 25OHD and dietary vitamin D intake by centre. Bergen had significantly higher 25OHD concentrations than all other centres (61 nmol/L), while the lowest 25OHD concentration was found in Paris (36 nmol/L). The proportion of vitamin D deficiency was 41% overall, and varied by centre with the lowest proportion in Bergen (26%), and the highest in Thessaloniki (48%). The proportion with insufficient vitamin D concentrations was 33% overall, lowest in Paris (21%) and highest in Bergen (44%). Dietary vitamin D intake varied by centre, with a two-fold difference from the lowest dietary intake of vitamin D observed to the highest. Thessaloniki had the lowest intake of dietary vitamin D (1.2 $\mu\text{g}/\text{day}$), whereas the highest intake of dietary vitamin D was found in Alicante (2.9 $\mu\text{g}/\text{day}$).

In univariable analysis, factors associated with seasonally adjusted 25OHD concentrations were age, sex, alcohol consumption, self-reported diabetes, oily fish intake, dietary vitamin D, supplements with fish liver oil, omega-3, and vitamin D (Table 3). In multivariable analysis, increasing age, self-reported diabetes and current smoking were associated with lower 25OHD concentrations and increasing dietary vitamin D, and supplement use with fish liver, omega-3 or vitamin D with higher concentrations of 25OHD (Table 4). Supplement use especially omega-3 was associated with the largest differences in 25OHD between users and non-users. There was no evidence of collinearity in the analyses between different types of supplements. The model explained 10.8% of the variance in 25OHD concentrations. Because of the much higher concentrations of 25OHD in Bergen, we carried out a further analysis excluding participants from Bergen and dropping omega-3 and vitamin D supplement use

(since few participants outside the Bergen centre used these supplements). In this model, age, diabetes and smoking remained significantly associated with lower 25OHD concentrations and, in addition to fish liver oil supplement, oily fish consumption >2 portions/week and UVB exposure were associated with higher 25OHD concentrations (Table 4). Results for dietary vitamin D were slightly attenuated compared to the previous model including Bergen. This model explained 7.2% of the variance in 25OHD concentrations. Differences in 25OHD were similar to those reported in the previous model including Bergen, for example around 5 nmol/L lower in participants with diabetes compared to participants with no reported diabetes in both models and 9 nmol/L higher with fish oil supplement use.

Discussion

Our study confirms findings from studies of European populations of low 25OHD concentrations and high proportions with vitamin D deficiency or insufficient levels (21). We observed that for serum 25OHD concentrations there was no north to south gradient, as would perhaps have been expected for vitamin D. Lower concentrations of vitamin D were expected in more northerly centres due to reduced UVB exposure during the year compared to more southern countries. Bergen despite its geographical location as one of the most northerly located had the highest level of 25OHD and the lowest prevalence of vitamin D deficiency. This finding has also been observed in other observational studies and systematic reviews (20,21,34,35). Hilger and colleagues investigated global 25OHD concentrations in several countries and age groups in a systematic review. While global 25OHD concentrations were approximately 53 nmol/L, it was observed in sub-analysis that older people in Sweden had comparatively higher 25OHD concentrations than any other older population in Europe (20). It can be hypothesised that Sweden and Norway would have similar dietary and cultural practices in addition to similar UVB exposure. Cashman and colleagues reported, based on 14 European population-based studies, that, in both adults and older adults, the prevalence of vitamin D deficiency was lower in more northerly latitude countries, such as Norway, Finland and Iceland, whereas countries in central Europe had a higher prevalence of vitamin D deficiency (21). It has been hypothesised that the high levels of 25OHD observed in Norway may be due to the high consumption of fish and food fortification of basic food items (34,35). Comparable to the current study, O'Neill and colleagues observed higher 25OHD concentrations in northern European countries, compared to central Europe during the winter months (36). The high proportion of omega-3 fish oil supplement users in Bergen (44%) is likely to be one of the sources of the high 25OHD concentrations. It is also notable that fish oil, which is the likely source of vitamin D in the Norwegian population, is considered a food

rather than a supplement (37). Omega-3 fish oils were strongly associated with seasonally adjusted 25OHD despite these supplements not containing vitamin D. We hypothesise but cannot confirm that the omega-3 fish oils consumed by participants in Bergen could be fortified with vitamin D. The use of omega-3 fish oil supplements was low in all other centres in the current study; in contrast fish liver oil supplement was more common in Belfast (24%). However the participants in the Bergen sample may have other characteristics that are associated with increased production of 25OHD₃ concentrations through UVB exposure such as paler skin (38). In contrast, Paris with the lowest 25OHD concentrations and the highest proportion of vitamin D deficiency had the lowest UVB exposures probably reflecting the lifestyle of a central urban population.

Age was negatively associated with 25OHD concentrations. Ageing can have a substantial effect on 25OHD concentrations. This is predominantly due to the reduced dermal capacity of older individuals to produce vitamin D₃. The capacity of the skin to produce vitamin D₃ is approximately 75% less in an older individual (>65 years) compared to an average 25 year old being exposed to the same amount of UVB radiation (39,40). Decreasing concentrations of 25OHD with increasing age may also be due to a change in behaviours, for example, older individuals tend to be outside less due to decreased ability to do physical activity and may wear more concealed clothing compared to their younger counterparts (41). Van Schoor and colleagues observed in a younger cohort (average age 60 ±3.0 years; n=738) that, for every year increase, 25OHD increased by 0.6 nmol/L, whereas in the older cohort (average age 76.0 ±6.7 years; n=1320), it was observed that for every year increase in age, 25OHD decreased by 1.0 nmol/L(42).

Smoking was negatively associated with serum 25OHD in the current study. This is in line with a study from a Danish population of 510 perimenopausal women aged 45-58 years. 50% of study participants were current smokers, and it was observed that smoking significantly

decreased serum 25OHD (43). Additionally, in a study of 612 older men of which 31% were current smokers, current smokers had significantly lower vitamin D than those who never smoked. This association showed a dose-response relationship (44). The method by which smoking influences serum 25OHD is currently unknown, and difficult to determine due to the large number of toxic chemical compounds. There is evidence to suggest that smokers have altered hepatic function (45), and therefore could influence 25OHD concentrations.

Dietary vitamin D intake in the current study was below the recommended adequate intake (AI) for European adults as set by the European Food Safety Authority (EFSA) (46). EFSA suggests that the AI for adults (>18 years of age) should be 15 µg/day. The highest observed intake of dietary vitamin D in the current study was in Alicante (2.9 µg/day), and the overall average for the total population was 2.0 µg/day. In contrast, the European Prospective Investigation into Cancer and Nutrition (EPIC) study observed a north/south gradient for dietary vitamin D intakes. The highest dietary vitamin D intake was observed in Sweden (7.6 µg/day), with the lowest observed in Italy (1.9 µg/day). Overall, the average dietary vitamin D intake across Europe in the EPIC study was 4.2 µg/day (47). While these intakes are still below the AI for European adults, they are higher than observed in the current study. This may be due to the inability of our dietary analysis to quantify dietary vitamin D from fortified foods. Fortified foods are an important source of dietary vitamin D in several countries. Mandatory fortification with vitamin D of margarine, butter and one type of skimmed milk is employed in Norway(48). Up until 2013 the UK had mandatory fortification of margarine with vitamin D, the current study was conducted between 2001-2002, and therefore margarine was subject to fortification. Voluntary fortification is also in use in the UK and France for food items such as dairy foods, cereals and vegetable oils (17,49). The remaining centres (Estonia, Italy, Spain and Greece) have no formal fortification practices (23,50,51).

This could suggest that our study has been unable to identify important sources of dietary vitamin D in habitual diets in several centres.

Lower serum 25OHD concentrations were associated with self-reported diabetes in the current study. Only a few studies have investigated the relationship between 25OHD and diabetes in an older population. Hirani and colleagues found that in people (>70 years) 25OHD concentrations of less than 50 nmol/L were associated with type 2 diabetes in confounder adjusted analysis (OR = 1.73, 95% CI = 1.04–2.86, p=0.03). 25OHD concentrations above 50 nmol/L were not significantly associated with type 2 diabetes(52). Park and colleagues in a 12 year longitudinal study of older people observed higher 25OHD concentrations (>75 nmol/L) had a lower hazard ratio for type 2 diabetes compared to lower 25OHD concentrations. There was an inverse dose-response gradient between 25OHD concentration and risk of diabetes; each 25 nmol/L increase was associated with a 0.64 hazard ratio for diabetes (53). These findings of an association between lower 25OHD and diabetes in an older population have important implications for health. Type 2 diabetes is a growing health problem (54) and, if compounded by deficiency of vitamin D, which has important roles in bone health and immune regulation (55,56), may potentially cause worse morbidity in this group. The association of 25OHD with type 2 diabetes is biologically plausible, as it has been suggested that vitamin D sufficiency may be required for optimal insulin action and secretion, which are two fundamental features of type 2 diabetes. The vitamin D receptor and 1- α -hydroxylase are both present in β -cells of the pancreas (57,58). *In-vitro* and *in-vivo* studies show that vitamin D receptor knockout mice, had impaired glucose induced insulin secretion, and the insulin secretory response only improved after vitamin D supplementation in both animals and humans. However, a causal effect remains to be demonstrated in human studies and cannot be determined from this class of a study(59).

The current study has several strengths and limitations. We investigated data from seven centres that spanned latitudes extending from north to south of Europe, and this encompassed a range of dietary habits and lifestyle behaviours. 25OHD₂ and 25OHD₃ were measured using the gold-standard method of measuring 25OHD (LC-MS/MS) using commercial calibrators and internal standards. Accuracy and precision of the assay was also maintained using the vitamin D external quality assurance system (DEQAS). The limitations of the current study include the retrospective and self-reported nature of lifestyle variables, including supplement use, smoking, alcohol, diabetes and CVD history. EUREYE had a 45% response rate and this may have resulted in a sample that were more health conscious and therefore we may be underestimating the prevalence of vitamin D deficiency. The use of UK food tables to analyse the dietary intake of all centres is a limitation, since country-specific food tables were not available for the majority of the countries involved in the EUREYE study. Furthermore, information on vitamin D supplement use was relatively limited, with only vitamin D supplement intake being included if reported; multivitamin use that may have included vitamin D was not included due to a lack of individual level data on the brands of supplements. Information on ethnicity and skin colour of the participants were also not available in the current study. As this is a cross-sectional observational study, we cannot conclude any casual or confirmatory effects of low 25OHD concentrations on type 2 diabetes.

In conclusion, we observed low concentrations of 25OHD across Europe in an older population group, an overall vitamin D deficiency of 41% and noted the lack of a north-south gradient. Our findings of the higher 25OHD concentrations in supplement users (omega -3 fish oil, fish liver oil, vitamin D) confirm those reported in other European studies (21) and support recommendations in the UK (17) and other European countries (35) for improving 25OHD concentrations. We were unable to fully assess the role of dietary vitamin D as we lacked information on vitamin D fortified foods.

Conflict of interest: The authors have nothing to disclose.

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UC, MR, JS, GS, LT, FT, JV and AEF designed and implemented the EUREYE study. CC, ISY, JW, AMcG formulated the current research question, JMcP carried out the laboratory analysis, CC and AEF analyzed the data. JW, AMcG and AEF supervised the work. CC wrote the manuscript. All authors revised the manuscript and approved the final draft. All authors have agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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- 1 **Fig. 1** Comparison of total 25OHD concentrations between seasons in the EUREYE
- 2 population and by centre
- 3 25OHD, 25-hydroxyvitamin D, nmol/L, nanomole per litre
- 4 Results are presented as geometric mean and 25th,75th quartiles
- 5