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C3-epimerization of 25-hydroxyvitamin D increases with increasing serum 25-hydroxyvitamin D levels and shows a high degree of tracking over time

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Abstract

Objective: Evaluate the effects of serum 25-hydroxyvitamin D (25(OH)D) levels, vitamin D binding protein (DBP) and genetic factors on C3-epimerization of 25(OH)D and follow the tracking of the epimer during one year.

Design: Cross-sectional and longitudinal study.

Methods: Data from eight previously conducted, Tromsø based studies (3 observational, 5 randomized controlled trials) were combined. 25(OH)D serum samples were re-analyzed with a LC-MS/MS method that also resolves and measures the metabolite C3-epi-25(OH)D3. Data on vitamin D binding protein (DBP) phenotype (based on single nucleotide polymorphisms (SNPs) rs4588 and rs7041) and genetic determinants for serum 25(OH)D (SNPs rs2282679, rs10741657, rs3829251 and rs6013897) were collected where available.

Results: 2219 subjects were included. Median (5th, 95th percentiles) baseline serum values of 25(OH)D3, C3-epi-25(OH)D3, and %-C3-epi-25(OH)D3 were 49.1 (22.1, 92.8) nmol/L, 2.3 (0.9, 6.0) nmol/L and 4.4 (2.7, 8.4) %, respectively. The highest baseline values were 230.5 nmol/L for 25(OH)D3, 79.7 nmol/L for C3-epi-25(OH)D3 and 48.2 % for %-C3-epi-25(OH)D3. There was a strong correlation between serum 25(OH)D3 and C3-epi-25(OH)D3. The %-C3-epi-25(OH)D3 value increased with increasing serum 25(OH)D3, but leveled off at ~7 % at a 25(OH)D3 concentration of ~120-140 nmol/L. There was a significant degree of tracking for %-C3-epi-25(OH)D3 (correlation coefficient rho between baseline and 1-year values 0.39, $P < 0.001$). The %-C3-epi-25(OH)D3 level was not related to serum DBP level, DBP phenotype nor to SNPs related to serum 25OH)D3 level. The serum 25(OH)D3 level could explain less than 3 % of %-C3-epi-25(OH)D3 variation.

Conclusions: There are considerable individual and reproducible differences in percent C3-epimerization of uncertain clinical importance.

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Introduction

The structure of nutritional forms of vitamin D became known in the 1930s and vitamin D`s role in calcium metabolism and musculoskeletal disease was thereafter established. In the 1960s it was determined that vitamin D had to be converted to become active, and nearly 10 years later the main circulating form 25-hydroxyvitamin D (25(OH)D) and the final active hormone 1,25-dihydroxyvitamin D ($1,25(OH)_2D$) were isolated and identified (1).

Today, the serum levels of 25(OH)D are used to evaluate a subject's vitamin D status, and depending on the assay used, may include both $25(OH)D_3$ derived from vitamin D_3 (cholecalciferol) and $25(OH)D_2$ derived from vitamin D_2 (ergocalciferol). As cholecalciferol is produced in the skin stimulated by sun exposure and also is the main nutritional and supplemental form, serum $25(OH)D_3$ levels are much higher than $25(OH)D_2$, the latter often being undetectable (1).

Recently it was discovered that vitamin D metabolites could be further metabolized through a C3-epimerization pathway (2, 3) that is found in many cell types (3, 4), and that the amount of C3-epi-$25(OH)D_3$ in infants could constitute up to 60 % of the total $25(OH)D_3$ concentration (5). However, the C3-epi-$25(OH)D_3$ form is not detected by immunoassays, only partly detected by competitive protein binding assays, and not separated from the non-epimeric form by most LC-MS/MS methods (6).

C3-epi-$25(OH)D_3$ and C3-epi-$1,25(OH)_2D_3$ bind, albeit relatively weak, to both the vitamin D binding protein (DBP) and the vitamin D receptor (VDR). At least in in-vitro experiments, the epimers appear to have calcemic, as well as non-calcemic effects, although to a much lesser extent than their non-epimeric counterparts (7). Because of this limited biological effect of C3-epi-$25(OH)D_3$ and the variability of the percent of $25(OH)D_3$ it constitutes in individuals (7-10), the clinical importance of distinguishing this variant from $25(OH)D_3$ is uncertain (9).

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Epidemiological studies on adults have shown serum levels of C3-epi-25(OH)D_3 to be strongly correlated to serum 25(OH)D_3 levels (9, 10), increase during summer months, be higher in males than in females, and decrease with expanding waist circumference (10). However, it is not known what regulates epimerization, nor what enzymes are involved.

It is unknown if DBP and genetic variants related to serum 25(OH)D levels likewise impact on the C3-epimer and, additionally, there is little data on the epimer's response to supplementation and tracking of its levels over time. We have recently performed several epidemiological, as well as randomized controlled trials (RCTs) with vitamin D supplementation and therefore had the opportunity to address these questions.

Materials and methods

In the present study, we have included data from eight Tromsø-based studies; three epidemiological studies and five RCTs. As part of the ODIN project (ODIN; www.odin-vitd.eu), - an EC sponsored project where individual participant data from observational and interventional data are meta-analyzed - serum samples from these studies were re-analyzed using a LC-MS/MS method at the Cork Centre for Vitamin D and Nutrition Research at University College Cork, Ireland. Full details of this LC-MS/MS method have been published elsewhere (10), but of note, in addition to 25(OH)D_3 and 25(OH)D_2 the method also resolves and measures the metabolite C3-epi-25(OH)D_3. The method's limit of detection (LOD) and limit of quantification (LOQ) for C3-epi-25(OH)D_3 in serum was 0.20 and 0.66 nmol/L, respectively. The Centre's LC-MS/MS method is certified by the CDC's *Vitamin D Standardization Certification Program*, which reports total 25(OH)D, 25(OH)D_3 and 3-epi-25(OH)D_3 (https://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf).

The eight studies have been described in detail before, and are summarized in short:

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- The Tromsø study 4th survey ("Tromsø 4") performed in 1994/1995 included 27 158 subjects (11), 7168 had serum 25(OH)D measured (12), and for standardization purposes 316 selected subjects had 25(OH)D re-analyzed at Cork (13).

- The Tromsø study 6th survey ("Tromsø 6") performed in 2007/2008 included 12 984 subjects (11), 12 817 had serum 25(OH)D measured, and for standardization purposes 340 selected subjects had 25(OH)D re-analyzed at Cork (14).

- Fit futures ("Fit Futures"), an adolescent population study and expansion of the Tromsø study, included 1038 subjects aged 15-18 years (15). 890 had serum 25(OH)D measured and for standardization purposes 171 selected subjects had 25(OH)D re-analyzed at Cork (14).

- The vitamin D and obesity study ("Obesity study") where 438 subjects with BMI 28.0 – 47.0 kg/m^2 were included and randomized to 40 000 IU vitamin D per week, 20 000 IU vitamin D per week or placebo for one year with weight loss as main endpoint (16). In the present study those given 40 000 and 20 000 IU were combined to one vitamin D group. Serum 25(OH)D was re-analyzed at Cork in 317 subjects who completed the study and had available serum samples.

- The vitamin D and insulin sensitivity study ("Clamp study") where 108 subjects with serum 25(OH)D < 40 nmol/L in Tromsø 6 were included and randomized to 40 000 IU vitamin D per week versus placebo for six months (17). Serum 25(OH)D was re-analyzed at Cork in 94 subjects who completed the study and had available serum samples.

- The vitamin D and bone density study ("Osteoporosis study") where 297 postmenopausal women aged 50 – 80 years and with a T-score in total hip or lumbar spine (L2-4) $\leq$ -2.0 were included and randomized to vitamin D 40 000 IU per week

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versus placebo for one year, with change in bone mass density (BMD) as primary endpoint. In addition, all subjects were given daily supplements with 1 g calcium and 800 IU vitamin D (18). Serum 25(OH)D was re-analyzed at Cork in 272 subjects who completed the study and had available serum samples.

- The vitamin D and depression study ("Depression study") where 243 subjects with serum 25(OH)D < 50 nmol/L in Tromsø 6 were randomized to vitamin D 40 000 IU per week versus placebo for six months with change in depression score as endpoint (19). Serum 25(OH)D was re-analyzed at Cork in 229 subjects who completed the study and had available serum samples.

- The vitamin D and prevention of diabetes study ("Diabetes study") where 511 subjects with impaired fasting glucose and/or impaired glucose tolerance were randomized to vitamin D 20 000 per week versus placebo for five years, and where development of type 2 diabetes (T2DM) was primary endpoint (20). Serum 25(OH)D was re-analyzed at Cork in 480 subjects who completed the first year of the study and had available serum samples.

Laboratory analyses

Serum DBP was analyzed as previously described by immunoassay using a polyclonal antibody (21). Genotyping was performed for selected SNPs previously shown to be related to serum 25(OH)D level: rs2282679, rs7041 and rs4588 at the *DBP/GC*gene; rs10741657 at the 25-hydroxylase gene (*CYP2R1*) involved in the conversion of vitamin D into 25(OH)D; rs3829251 at the 7-dehydrocholesterol (7-DHC) reductase/NAD synthetase 1 gene (*DHCR7/NADSYN1*) responsible for the availability of 7-DHC in the skin; and rs6013897 in the 24-hydroxylase gene (*CYP24A1*) involved in the degradation of 25(OH)D (22). Genotyping of rs4588 and rs7041 was used to determine DBP phenotype (23). The

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genotyping was peformed by KBioscience (http://www.kbioscience.co.uk) using KASP (KBioSience Allele-Specific Polymorphism) SNP genotyping system, a competitive allele-specific polymerase chain reaction, which has been described in detail before (22).

Statistical analyses

Normal distribution was evaluated with visual inspection of histograms, and by assessing kurtosis and skewness. Only BMI and DBP were normally distributed; serum 25(OH)D_3, C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3 attained normal distribution after Lg transformation and were used as such where appropriate.

For SNP analyses, genotype frequencies were analyzed with Hardy-Weinberg equilibrium calculator (24) with no bias detected. Correlations were performed with Spearman's rho and comparisons between groups were made on Lg-transformed data using ANOVA with Bonferroni correction for DBP phenotypes and linear trend across genotypes for SNPs. Mann-Whitney U test was used for comparing median %-C3-epi-25(OH)D_3 between 25(OH)D_3 groups. In the Diabetes study, a linear regression model was used for determining predictors of serum 25(OH)D_3, C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3, with covariates as described in the text. %-C3-epi-25(OH)D_3 was calculated as (serum C3-epi-25(OH)D_3 concentration x 100)/(serum C3-epi-25(OH)D_3 concentration + serum 25(OH)D_3 concentration). Delta values were achieved as (endpoint values) - (baseline values).

$P < 0.05$ (two-tailed) was considered statically significant. Data are presented as mean ± SD for normally distributed values and as median (5^{th}, 95^{th} percentiles) for non-normally distributed values. All statistical analyses were performed using IBM SPSS version 24 software.

Ethics

All eight studies were approved by the Regional committee for Medical Research Ethics and the five intervention studies by the Norwegian Medicines Agency.

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Results

A total of 2219 subjects (1055 males) were included; their baseline and endpoint characteristics in relation to the original studies shown in Table 1. Where ethnicity could be determined 98.1 % of the participants were shown to be of Norwegian descent, the second largest ethnic group being Sami at 1.1 %. For all studies taken together, the median (5%, 95%) baseline values were: serum $25(OH)D_3$ 49.1 (22.1, 98.8) nmol/L, C3-epi-$25(OH)D_3$ 2.3 (0.9, 6.0) nmol/L, %-C3-epi-$25(OH)D_3$ 4.4 (2.7, 8.4) %. The highest baseline values were 230.5 nmol/L for $25(OH)D_3$, 79.7 nmol/L for C3-epi-$25(OH)D_3$ and 48.2 % for %- C3-epi-$25(OH)D_3$. For subjects with serum $25(OH)D_3 < 50$ nmol/L (the ones where misclassification due to epimer form could be of clinical importance), only 2.4 % had %-C3-epi-$25(OH)D_3 >$ 10 % and only 0.4 % had %-C3-epi-$25(OH)D_3 > 15$ %, the highest being 19.5 %. There was no correlation between $25(OH)D_2$ and %-C3-epi-$25(OH)D_3$ and thus, as vitamin D_2 does not seem to be related to the process of vitamin D_3 epimerization it is not discussed further in the article.

Correlations

As expected, there were strong correlations between $25(OH)D_3$, C3-epi-$25(OH)D_3$ and %-C3-epi-$25(OH)D_3$ at baseline when all 8 studies were pooled together (Table 2), which also remained when analyzed separately (data not shown). Similarly, there was a strong correlation between delta values when the five RCTs were pooled together (Supplemental Table 1), as well as when analyzed separately (data not shown).

In the Diabetes study, using a linear regression model with age, BMI, gender and $25(OH)D_3$ as covariates, only $25(OH)D_3$ significantly predicted Lg serum C3-epi-$25(OH)D_3$ ($R^2 = 0.56$, standardized beta coefficient $= 0.75$, $P < 0.001$) and Lg %-C3-epi-$25(OH)D_3$ ($R^2 =$ 0.03, standardized beta coefficient $= 0.17$, $P < 0.001$).

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The increase in C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3 with rising serum 25(OH)D_3 concentration was most apparent when all studies were merged together, including both baseline and post-intervention values. The epimer level showed a nearly linear increase with rising 25(OH)D_3 levels, with no sign of being affected by extreme 25(OH)D_3 values on either side of the scale. %-C3-epi-25(OH)D_3 levels on the other hand displayed a more S-shaped pattern in relation to 25(OH)D_3, as shown in Figure 1 and Supplemental Table 2. Thus, the %-C3-epi-25(OH)D_3 remained low (~4 %) in the vitamin D insufficient/deficient groups (< 50 nmol/L), subsequently increasing before leveling off at ~7 % at a serum 25(OH)D_3 concentration of ~120-140 nmol/L. Accordingly, in subjects with serum 25(OH)D_3 > 140 nmol/L (n = 20), there was no significant correlation between %-C3-epi-25(OH)D_3 and 25(OH)D_3 (rho = 0.14, P = 0.83). Subjects with serum 25(OH)D_3 > 120 nmol/L had significantly higher serum %-C3-epi-25(OH)D_3 than to those with a 25(OH)D_3 level <50 nmol/L, as well as those with serum 25(OH)D_3 <100 nmol/L ($P < 0.05$).

Tracking

To evaluate tracking of serum 25(OH)D_3, C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3, subjects randomized to placebo in the Obesity and Diabetes studies (where blood samples were drawn at baseline and after 1 year) (n=345), were analyzed. The respective correlation coefficients between baseline and 1 year values for serum 25(OH)D_3, C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3 were all highly significant, with rho-values at 0.76, 0.47, 0.39, respectively ($P < 0.001$).

Since both C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3 strongly correlate with the 25(OH)D_3 level, we examined if their tracking could simply be the result of co-variation with the latter. A subgroup analysis was therefor made, selecting subjects within a narrow range of baseline serum 25(OH)D_3 (45 - 55 nmol/L) and with minimal change in serum 25(OH)D_3 from baseline to 1 year (delta 25(OH)D_3 > -5nmol/L and < 5nmol/L, n=32 (5 subjects in the

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Obesity and 27 in the Diabetes study)). Although these subjects had relatively similar $25(OH)D_3$ levels at baseline, there was a wide distribution of baseline serum C3-epi-$25(OH)D_3$ concentrations and %-C3-epi-$25(OH)D_3$ levels, with a high degree of tracking in the 1 year values (Figures 2 and Supplemental Figure 1). In this subgroup the correlation coefficients rho for baseline and 1 year values were 0.70 for C3-epi-$25(OH)D_3$ and 0.75 for %-C3-epi-$25(OH)D_3$.

DBP

DBP was measured at baseline in the Diabetes study (n = 471) and correlated significantly with $25(OH)D_3$ (rho 0.20, $P < 0.001$) and C3-epi-$25(OH)D_3$ (rho 0.10, $P = 0.029$). For %-C3-epi-$25(OH)D_3$ there was no significant correlation with DBP (rho -0.04, $P = 0.33$). In linear regression models with age, BMI and gender as covariates, only the relation between DBP and $25(OH)D_3$ remained significant. Similarly, there were significant differences in serum $25(OH)D_3$ and C3-epi-$25(OH)D_3$ between DBP phenotypes, whereas %-C3-epi-$25(OH)D_3$ was unrelated (Table 3). To evaluate if the association between C3-epi-$25(OH)D_3$ and DBP phenotype was due to co-variation with $25(OH)D_3$, we used a general linear model with C3-epi-$25(OH)D_3$ as dependent variable, DBP phenotypes Gc-1S/Gc-1S and Gc-2/Gc-2 as fixed factors and serum $25(OH)D_3$ as covariate, finding the relation between these DBP phenotypes and C3-epi-$25(OH)D_3$ no longer significant (data not shown).

Effects of genotypes related to serum 25(OH)D levels

Serum $25(OH)D_3$ was found significantly related to SNPs rs10741657 (*CYP2R1* gene) and rs2282679 (*DBP/GC* gene) at both baseline and endpoint. Serum $25(OH)D_3$ correlated additionally to rs6013897 (*CYP24A1* gene) at baseline value, whereas the relation to rs3829251 (*DHCR7/NADSYN1* gene) was not significant (Table 4). C3-epi-$25(OH)D_3$ had significant association to rs2282679, with a statistically significant linear trend across the phenotypes. No significant relations were found between %-C3-epi-$25(OH)D_3$ and any of

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these SNPs. The significant linear trend for C3-epi-25(OH)D_3 across the rs2282679 genotypes became non-significant when including 25(OH)D_3 as a covariate in the regression model, indicating that similar to DBP the correlation between C3-epi-25(OH)D_3 and the SNP was a result of co-variation with 25(OH)D_3.

Discussion

In the present study we have found low median serum C3-epi-25(OH)D_3 levels, strong correlations between serum 25(OH)D_3, C3-epi-25(OH)D_3 and %-C3-epi-25(OH)D_3, a high degree of tracking for %-C3-epi-25(OH)D_3, and no influence on %-C3-epi-25(OH)D_3 by serum DBP level, DBP phenotype nor SNPs related to serum 25(OH)D_3 levels.

So far, most studies on vitamin D epimerization in adults have reported the mean C3-epi-25(OH)D_3 percentage to be low (10, 25), but also that a few subjects have remarkably high C3-epimer concentrations (7, 9, 10). Similarly, we found the median %-C3-epi-25(OH)D_3 at baseline to be 4.3%, but with a wide range from 0 to 48 %. Furthermore, when selecting vitamin D deficient subjects (serum 25(OH)D_3 levels < 50 nmol/L)(26), who would be the ones with highest risk of vitamin D status misclassification, only 2.4 % had %-C3-epi-25(OH)D_3 levels above 10 %. Accordingly, regardless of whether the assay used detects and/or differentiates these vitamin D metabolites, and of whether the C3-epi-25(OH)D_3 is biologically active or not (2, 7), very few adults risk misclassification because of the epimer form. These results are in agreement with the findnings of Cashman et al. (10). It should be noted that this may not be true for infants, as the percentage epimer in the very young has been reported as overall higher than in adults (5, 7).

There was a strong and positive correlation between 25(OH)D_3 and C3-epi-25(OH)D_3, with rising percentage of the epimer the higher the 25(OH)D_3, as also reported by Cashman et al. (10). However, as shown in Figure 1, the increase in %-C3-epi-25(OH)D_3 appeared to start first at a median serum 25(OH)D_3 concentration of ~50-60 nmol/L and leveled off at a median

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25(OH)D_3 concentration of ~120-140 nmol/L. To our knowledge this has not been described before, as both Cashman et al. and Engelman et al. found a more linear realtionship in their data, possibly due to fewer subjects with high serum 25(OH)D_3 levels (10, 25). Our findings could indicate that the putative epimerization enzyme is switched on or activated by increasing serum 25(OH)D_3 concentrations, and becomes saturated at a point corresponding to an upper or maximum physiological 25(OH)D_3 level. If the epimeric form is less active than the non-epimeric form, this could be considered a protective mechanism against too high levels and possibly unwanted effects of vitamin D (in addition to the catabolism of 25(OH)D_3-induced by 24-hydroxylation); a process that could be parallel to the conversion of thyoxine (T4) to inactive reverse triiodothyronine (rT3) when there is a need to reduce the metabolic rate (27). This is of course highly speculative and needs both in-vitro and in-vivo confirmation, in addition to being difficult to demonstrate before the enzyme responsible for epimerization is discovered. Furthermore, it has been shown that a substantial part of C3-epi-25(OH)D_3 must have endogenous origin (10). Since epimerization may occur before 25-hydroxylation, and may also differ in various tissues (4), it would in view of our hypothesis be highly interesting to study the degree of epimerization of vitamin D in epidermal cells in relation to sun exposure.

There is a known high degree of tracking of serum 25(OH)D_3 over time (12). This has also been described for the epimeric form (9, 28), and is confirmed in our study. In addition, we show for the first time, that there is a considerable degree of tracking for %-C3-epi-25(OH)D_3 that cannot be attributed to simple co-variation with serum 25(OH)D_3. This was clearly shown when subjects with baseline serum 25(OH)D_3 within a narrow range and only a small change in serum 25(OH)D_3 at the one-year follow-up were analyzed separately. In this group of 32 subjects, there was a high degree of tracking in spite of a wide range of %-C3-epi-25(OH)D_3 values at baseline. Accordingly, the percent epimer truly differs between

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individuals and cannot be ascribed to inaccurate measurements magnified by the use of a ratio for its determination. One might therefore assume that the percentage of epimerization also is regulated by other factors than the serum 25(OH)D_3 level. This was confirmed in the Diabetes study where a linear regression model including age, gender, BMI and serum 25(OH)D_3 could explain 56 % of the variation in serum C3-epi-25(OH)D_3, but only 3 % of the variability of %-C3-epi-25(OH)D_3. Therefore, more influential variables that determine an individual's epimer percentage remain to be discovered, and DBP and genetic factors appeared as likely candidates.

The binding coefficient between 25(OH)D and DBP varies with DBP phenotype, and if the same is the case for the epimer form, this would influence how much epimer circulates in free, unbound form. As it is likely that the free form is more prone to degradation than the bound form, DBP phenotype could in theory influence %-C3-epi-25(OH)D_3 levels. In the Diabetes study where DBP was measured, we found the expected relations between DBP phenotype and serum 25(OH)D_3 (23), which due to co-variation was also seen for C3-epi-25(OH)D_3. However, we found no significant relation between DBP phenotype and %-C3-epi-25(OH)D_3.

It has been shown that ethic background influences both DBP and 25(OH)D_3 levels, and up to 79.4 % of the variation in DBP level may be explained by genetic factors (29). Race is therefore important to consider when drawing conclusions on 25(OH)D_3 and DBP levels. Subjects enrolled in our data were ethnically homogenous, with 98,1 % of the subjects with measured DBP phenotype (Diabetes study) being of Norwegian ethnicity, followed by the indigenous Sami population at 1.1 %. We therefore did not do subgroups analysis based on ethnicity. However, it can be noted that the most predominant phenotype in our cohort (Gc-1S/Gc-1S) is consistent with Powe's findings for their Caucasian population.

In search of genetic factors, we analysed SNPs known to affect levels of serum

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25(OH)D_3. We found the expected relations between serum 25(OH)D_3 and the selected SNPs (except for rs3829251, probably due to lack of power), and a similar pattern was seen for serum C3-epi-25(OH)D_3 in relation to SNP rs2282679. Analogous to that seen for DBP, this relation between C3-epi-25(OH)D_3 and rs2282679 was due to co-variation with 25(OH)D_3. And similarly, there was no relation between the analyzed SNPs and %-C3-epi-25(OH)D_3, the genetic factors influencing the epimerization remaining to be found.

The main weakness of our study is that we analyzed pooled data from eight separate studies. Our results should therefore be viewed with caution. The studies were neither separately, nor when pooled together, representative for the general population, and we therefore did not focus on epimerization effects of gender, age, BMI and similar variables, that we believed could be influenced by this. On the other hand, pooling the data and including both baseline and post-interventional values, gave us power to detect relations between vitamin D metabolites not described before.

In conclusion, we have found great and reproducible individual differences in percent C3-epimerization. These differences can only to a minor degree be explained by the corresponding serum 25(OH)D_3 levels. The main regulators and clinical importance (if any) of this epimerization process are still to be determined.

Declaration of interest

The authors have no conflicts of interest that could be perceived as prejudicing the impartiality of the presented work.

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Author contributions

Conception and design: JMK and RJ. Acquisition of the data: RJ, GG, KDC. Analysis and interpretation of the data: JMK, RJ, GG, KDC, KD, ZS, EK. Drafting the article: JMK and RJ. Revising it critically for important intellectual content: JMK, RJ, GG, KDC, DK, ZS, EK. Final approval of the versions to be published: JMK, RJ, GG, KDC.

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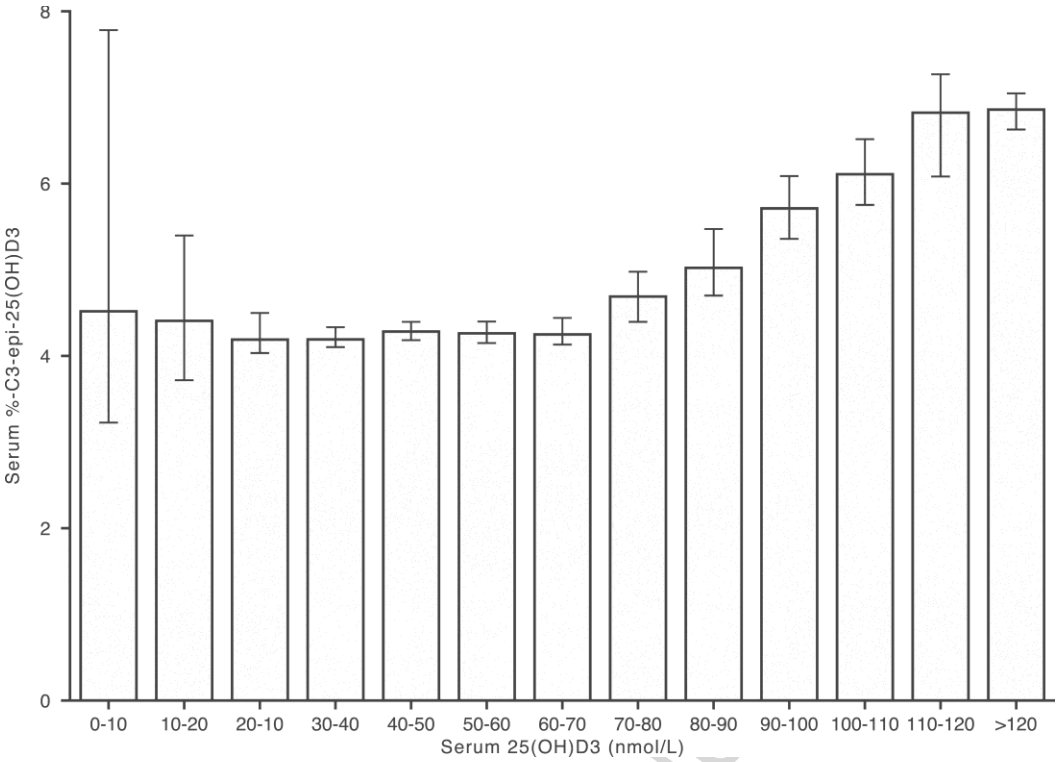


Fig: 1

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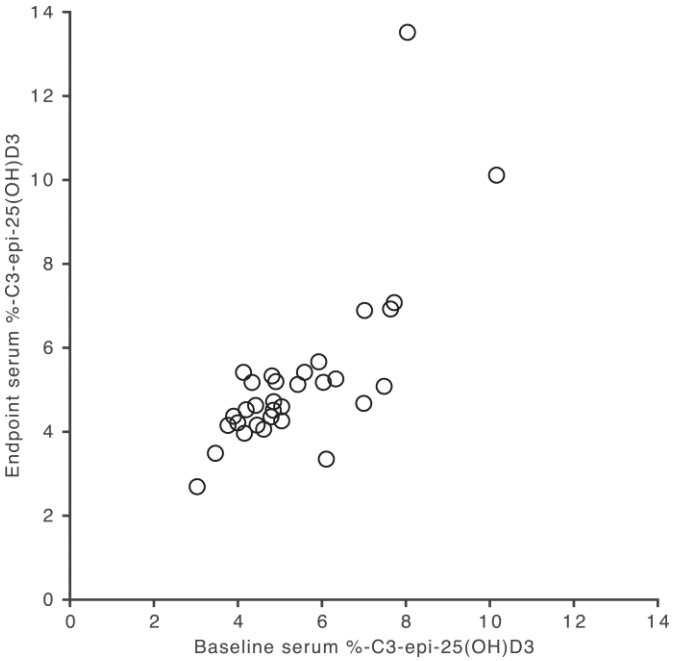


Fig 2:

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Table 1. General characteristics of the individual studies.

	Study							
	Tromsø 4	Tromsø 6	Fit Futures	Obesity	Clamp	Osteoporosis	Depression	Diabetes
	n = 316	n = 340	n = 171	n = 317	n = 94	n = 273	n = 229	n = 480
Intervention in Vitamin D group (IU/week)	-	-	-	40 000 - 20 000	40 000	40 000	40 000	40 000
Vitamin D group/placebo group (n/n)	-	-	-	212/105	49/45	135/138	120/109	240/240
Males (%)	66.1	56.5	52.6	37.5	52.1	0	44.1	61.5
Age (years)	58	60	16	50	51	62	51	63
	(38, 69)	(40, 78)	(16, 19)	(31, 66)	(42, 69)	(52, 75)	(38, 70)	(46, 76)
Smokers (%)	51.3	49.1	25.1	19.2	3.2	23.4	38.9	19.8
BMI (kg/m^2)	25.5	26.4	21.3	33.9	26.8	24.2	27.8	29.6
	(20.3, 32.4)	(19.8, 33.8)	(17.6, 30.7)	(29.3, 41.7)	(22.2, 31.9)	(20.2, 30.7)	(21.6, 35.1)	(23.3, 37.7)
Serum 25(OH) D_3 baseline	50.6	62.1	32.8	45.7	35.8	63.7	39.0	52.6
(nmol/L)	(24.1, 93.4)	(24.5, 141.2)	(9.6, 89.1)	(21.0, 83.3)	(17.3, 56.2)	(29.8, 96.7)	(22.7, 67.8)	(28.8, 85.2)
Serum 25(OH)	-	-	-	89.9	89.1	107.1	89.0	73.8

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	Study							
	Tromsø 4	Tromsø 6	Fit Futures	Obesity	Clamp	Osteoporosis	Depression	Diabetes
D_3 endpoint								
(nmol/L)				(29.9, 155.2)	(23.4, 161.1)	(60.1, 212.9)	(28.0, 163.9)	(34.0, 121.7)
Serum C3-epi-25(OH) D_3 baseline	2.0	2.3	1.7	2.7	1.4	2.6	1.7	2.6
(nmol/L)	(0.7, 5.9)	(0.8, 12.4)	(0.4, 6.2)	(1.1, 5.0)	(0.4, 3.1)	(1.3, 5.5)	(0.9, 3.3)	(1.2, 6.1)
Serum C3-epi-25(OH) D_3 endpoint	-	-	-	5.2	6.8	6.9	6.4	4.7
(nmol/L)				(1.1, 12.0)	(0.7, 13.4)	(2.6, 18.8)	(1.3, 14.2)	(1.5, 10.9)
Serum %-C3-epi-25(OH) D_3 baseline	3.8	3.7	5.0	5.1	3.7	4.2	4.2	4.6
(%)	(2.3, 7.0)	(2.0, 8.6)	(3.5, 7.8)	(3.2, 11.3)	(1.8, 6.9)	(2.98, 6.5)	(2.8, 6.6)	(3.1, 8.6)
Serum %-C3-epi-25(OH) D_3 endpoint	-	-	-	5.4	6.0	5.8	6.1	5.6
(%)				(3.2, 8.2)	(2.7, 10.0)	(3.6, 10.0)	(3.3, 10.7)	(3.3, 10.8)

The data are shown as % or median (5th, 95th percentile)

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Table 2. Correlations between baseline serum values for all studies pooled together (n = 2219).

	$25(OH)D_3$	$25(OH)D_2$	C3-epi-$25(OH)D_3$	%-C3-epi-$25(OH)D_3$
$25(OH)D_3$		-0.19**	0.77**	0.05*
$25(OH)D_2$	-0.19**		-0.15**	-0.02
C3-epi-$25(OH)D_3$	0.77**	-0.15**		0.63**
%-C3-epi-$25(OH)D_3$	0.05*	-0.02	0.63**	

The data show Spearman`s correlation coefficient rho.
* $P < 0.05$; ** $P < 0.001$

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Table 3: Serum $25(OH)D_3$, C3-epi-$25(OH)D_3$ and %-C3-epi-$25(OH)D_3$ at baseline in relation to vitamin D binding protein phenotypes in the diabetes study (n=471).

DBP phenotype		Count	$25(OH)D_3$	C3-epi-$25(OH)D_3$	%-C3-epi-$25(OH)D_3$
		(n)	(nmol/L)	(nmol/L)	(%)
Gc-1S/Gc-1S	(GC/GC)	146	54.2 (28.9, 86.9)	2.7 (1.3, 5.6)	4.6 (3.3, 7.9)
Gc-1S/Gc-1F	(GC/TC)	124	55.2 (32.6, 88.7)	2.7 (1.3, 6.2)	4.6 (3.2, 8.4)
Gc-1S/Gc-2	(GC/TA)	119	53.2 (30.2, 83.8)	2.6 (1.1, 6.3)	4.9 (2.9, 10.0)
Gc-1F/Gc-1F	(TC/TC)	19	47.8 (24.7, 77.1)	2.1 (1.0, 6.4)	4.3 (2.9, 7.9)
Gc-1F/Gc-2	(TC/TA)	39	50.3 (21.1, 77.0)	2.3 (1.0, 7.1)	4.5 (2.4, 9.2)
Gc-2/Gc-2	(TA/TA)	24	43.0 (20.4, 66.4)*	1.8 (0.9, 4.9)*	4.8 (3.2, 7.4)

Data are shown as median (5^{th}, 95^{th} percentile)
*P < 0.01 vs Gc-1S/Gc-1S, ANOVA using Lg transformed values

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Table 4: Serum levels of $25(OH)D_3$ and %-C3-epi-$25(OH)D_3$ at baseline and endpoint in relation to SNPs related to serum 25(OH)D3 levels in genotyped subjects

	Count	$25(OH)D_3$ baseline	$25(OH)D_3$ endpoint	%-C3-epi-$25(OH)D_3$ baseline	%-C3-epi-$25(OH)D_3$ endpoint
	(n)	(nmol/L)	(nmol/L)	(%)	(%)
rs3829251 (DHCR7/NADSYN1)					
Homozygous major (GG)	555	50.8 (24.6, 87.8)	86.5 (32.8, 179.8)	4.4 (2.9, 7.9)	5.8 (3.3, 10.5)
Heterozygous (CA)	412	48.3 (24.0, 83.4)	83.1 (30.9, 172.3)	4.3 (2.9, 7.4)	5.6 (3.3, 10.7)
Homozygous minor (AA)	82	50.4 (28.8, 91.9)	82.5 (34.3, 160.7)	4.1 (2.9, 7.9)	5.2 (3.3, 10.6)
rs10741657 (CYP2R1 gene)					
Homozygous major (GG)	458	48.6 (24.2, 85.6)	79.1 (34.3, 170.4)	4.2 (2.7, 8.0)	5.8 (3.2, 11.4)
Heterozygous (GA)	645	51.1 (23.6, 86.7)	84.2 (31.1, 163.6)	4.2 (2.8, 7.3)	5.7 (3.2, 10.2)
Homozygous minor (AA)	259	51.2 (25.5, 97.3) **	92.4 (32.2, 191.1) *	4.2 (2.7, 7.3)	5.8 (3.5, 9.8)
rs2282679 (GC/DBP gene)					
Homozygous major (AA)	745	53.7 (26.9, 93.4)	96.0 (34.0, 185.6)	4.2 (2.8, 7.3)	5.6 (3.3, 10.6)
Heterozygous (CA)	535	47.5 (23.6, 82.3)	86.4 (32.0, 164.1)	4.2 (2.7, 8.0)	5.8 (3.2, 10.9)
Homozygous minor (CC)	83	40.1 (21.9, 72.9) **	72.3 (26.9, 147.7) **	4.2 (3.0, 7.3)	5.7 (3.2, 9.6)
rs6013897 (CYP24A1 gene)					
Homozygous major (TT)	760	50.6 (26.6, 91.3)	96.0 (32.8, 178.7)	4.2 (2.7, 7.3)	5.7 (3.4, 10.2)
Heterozygous (TA)	516	49.6 (22.8, 84.9)	86.4 (32.0, 168.8)	4.2 (2.7, 7.7)	5.8 (3.1, 10.8)
Homozygous minor (AA)	79	49.0 (21.9, 88.4) *	72.3 (27.6, 176.0)	4.4 (2.6, 9.9)	6.2 (3.2, 10.6)

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Data shown as median (5^{th}, 95^{th})
*P <0.05; ** P <0.01 for linear trend across genotypes using Lg transformed values

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Highlights

- There is a significant degree of tracking for %-C3-epi-25(OH)D_3
- %-C3- epi-25(OH)D_3 level is unrelated to serum DBP level and DBP phenotype
- %-C3- epi-25(OH)D_3 level is unrelated to SNPs related to serum 25OH)D_3
- Serum 25(OH)D_3 level explains less than 3 % of %-C3-epi-25(OH)D_3 variation

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