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Serum levels of 25-hydroxyvitamin D and the CYP3A biomarker 4β-hydroxycholesterol in a high-dose vitamin D supplementation study

Linda Björkhem-Bergman, Hanna Nylén, Anna-Carin Norlin, Jonatan D Lindh, Lena Ekström, Erik Eliasson, Peter Bergman, Ulf Diczfalusy.

Karolinska Institutet, Department of Laboratory medicine, Divisions of Clinical Pharmacology (LBB, JDL, LE, EE), Clinical Chemistry (HN, UD), Clinical Immunology (ACN), and Clinical Microbiology (PB) and Department of Medicine, Center for Infectious Medicine (PB).
Karolinska University Hospital Huddinge, SE-141 86 Stockholm, Sweden.

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Running Title: Vitamin D and the CYP3A biomarker 4β-hydroxycholesterol

Corresponding author:
Linda Björkhem-Bergman (MD, PhD)
Division of Clinical Pharmacology C1-68,
Karolinska University Hospital, Huddinge
Karolinska Institutet, 141 86, Stockholm, Sweden
e-mail: linda.bjorkhem-bergman@karolinska.se
Tel: +46 8 585 839 66
Fax: +46 8 585 810 70

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Abbreviations used:

Cytochrome P450-enzyme 3A4, CYP3A4; vitamin D receptor gene, VDR; C-reactive Protein, CRP.

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Abstract

The primary aim was to study the relationship between individual serum levels of 25-hydroxyvitamin D and 4β-hydroxycholesterol, which is an endogenous biomarker of the drug-metabolising CYP3A enzymes. In addition, the relationship between this biomarker and inflammation, measured as C-reactive protein (CRP), was investigated. Serum samples were used from a recently performed clinical trial in patients with antibody deficiency or increased susceptibility to respiratory tract infections that were randomised to either placebo or high dose (4000 IU/day) vitamin D for 12 months. 116 patients were included in the final analyses and serum samples collected 6 months after study start were analysed. At this time point, 25-hydroxyvitamin D levels were found to range between 10-284 nmol/L. Individual levels of 25-hydroxyvitamin D as well as CRP were compared with 4β-hydroxycholesterol levels. In addition, all participants were genotyped for two polymorphisms (Taq1 and Foq1) in the vitamin D receptor gene (VDR). There was no significant correlation between individual serum levels of 25-hydroxyvitamin D and 4β-hydroxycholesterol. However, a moderate, but statistically significant, negative correlation between CRP and 4β-hydroxycholesterol levels was observed. This study in patients with highly variable serum levels of 25-hydroxyvitamin D could not reveal any relationship between vitamin D and 4β-hydroxycholesterol, an endogenous biomarker of CYP3A activity. However, the negative correlation between CRP and 4β-hydroxycholesterol supports earlier experimental results that inflammation may suppress hepatic CYP3A activity, a finding of potentially high clinical relevance that warrants further exploration.

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Introduction

The cytochrome P450-enzyme 3A4 (CYP3A4) is the most important human drug-metabolizing enzyme with regards to number of different drug substrates (Daly, 2006). The activity of this enzyme is known to show a significant variability not only between different individuals but also within the same individual at different time points. The reason for this variability is not fully understood but clearly, environmental factors should be involved to explain fluctuations in the individual CYP3A4 activity over time.

Vitamin D is synthesized in the skin under influence of UVB-light and is further undergoing two hydroxylation steps, which results in active vitamin D (1,25-dihydroxyvitamin D_3). This molecule binds to the vitamin D receptor (VDR) and a nuclear receptor partner. The heterodimer complex binds to vitamin D response elements (VDRE) in promoters of several genes, including CYP3A4 (Lindh et al., 2012). In cell experiments it has been shown that 1α,25-dihydroxyvitamin D_3 up-regulates the expression of the CYP3A4 gene in the human colon carcinoma cell line Caco-2 (Schmiedlin-Ren et al., 1997). This up-regulation results in increased metabolism of CYP3A4 drug substrates. The synthesis of vitamin D is dependent on exposure to UVB-light and the plasma level of 25-hydroxyvitamin D therefore exhibits seasonal variation, especially in countries like Sweden with great differences in sun-light exposure during summer and winter (Landin-Wilhelmsen et al., 1995; Virtanen et al., 2011). Taken together, we hypothesized that CYP3A4 expression and the corresponding drug-metabolising capacity might display cyclic changes over the different seasons. Indeed, in a retrospective study on a large patient material from therapeutic drug monitoring, we were able to show such cyclic, seasonal variability in blood levels of the important immunosuppressants tacrolimus and sirolimus, known to be metabolised by CYP3A4 (Lindh et al., 2011). Significantly lower concentration-to-dose ratios were evident during the months of peak vitamin D levels, July-September, as compared to December through February (Lindh et al., 2011). This original finding raised several important questions, for example whether the individual vitamin D-status is predictive of CYP3A4-activity, and if treatment of patients with vitamin D results in higher CYP3A4-dependent drug metabolism.

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4β-hydroxycholesterol is specifically formed by CYP3A-catalyzed metabolism of cholesterol and the serum level of this metabolite has been proposed to be useful as an endogenous marker of CYP3A activity (Diczfalusy et al., 2008). To study a possible correlation between the levels of 25-hydroxyvitamin D and 4β-hydroxycholesterol, we used material from a recent study performed at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden (Bergman et al., 2012). In this double-blind, placebo-controlled study 140 patients were randomised to receive placebo or oral vitamin D 4000 IU / day for 12 months. In this material, marked interindividual differences in the level of 25-hydroxyvitamin D was evident (10-284 nmol/ L), which was relevant for further studies on the relationship between 25-hydroxyvitamin D and CYP3A-activity. In addition, different gene polymorphisms known to affect the function of VDR had been determined in the subjects. Furthermore, this study material included patients with high frequency of infections, offering an opportunity to study whether acute inflammation, measured as C-reactive protein (CRP) impacts on the CYP3A biomarker. It has previously been shown in cell and animal experiments that infections and inflammation can down-regulate CYP3A4 expression (Morgan et al., 2008; Morgan, 2009) and a recent study in hemodialysis patients indicated a correlation between CYP3A4-dependant alprazolam 4-hydroxylation, and CRP. (Molanaei et al., 2012).

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Material and Methods

Study cohort

Serum samples were retrieved from a recently performed prospective, randomized, double-blind placebo-controlled study of vitamin D_3 supplementation in patients with an increased susceptibility to respiratory tract infections, described in details elsewhere. The study was approved by the regional Ethical Review Board and the Swedish Medical Products Agency, registered at www.clinicaltrials.gov (NCT01131858) and performed in accordance with the declaration of Helsinki. Patients at the Immunodeficiency Unit, Karolinska University Hospital, Huddinge, Sweden, were included between March 2010 and June 2010. Inclusion criteria were age 18–75 years and an increased susceptibility to respiratory tract infections defined as > 42 days with symptoms of respiratory tract infection during a 12 months period prior to study inclusion.

One hundred and forty patients were randomized to 12 months' treatment with oral vitamin D_3 (Vigantol®, 4000 IU/day) or placebo oil. 120 patients completed the study. In the present study we analyzed the serum samples collected 6 months from study start since that was the time point with the greatest observed difference in 25-hydroxyvitamin D-levels. Here, 117 patient serum samples were available but in one subject a very high 4β-hydroxycholesterol level (394 ng/mL) was observed. This was considered to result from on-going carbamazepine treatment and therefore excluded from the analysis. Eventually, 116 patient samples were included in the final analysis of this study, 58 from the vitamin D group and 58 from the placebo group. There were 85 women and 31 men of which 14 men where in the vitamin D-group and 17 men in the placebo group. The mean age was 54 years.

Two SNPs Taq1 (rs731236) and Foq1 (rs2228570) in the vitamin D receptor (VDR) gene were analyzed in all participants as previously described (Bergman et al., 2012). These gene polymorphisms have previously been shown to affect the function of VDR and the outcome of vitamin D supplementation (Aslan et al., 2011; Martineau et al., 2011).

Measurement of 25-hydroxyvitamin D, 4β-hydroxycholesterol and CRP

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Levels of 25-hydroxyvitamin D in serum were determined by a commercial immunochemical method, LIAISON 25 OH Vitamin D TOTAL Assay (DiaSorin S.p.A, Saluggia, Italy) at the Department of Clinical Chemistry, Karolinska University Hospital.

Serum 4β-hydroxycholesterol was measured by isotope dilution gas chromatography – mass spectrometry using hexadeuterium labeled 4β-hydroxycholesterol as internal standard as described elsewhere (Bodin et al., 2001; Diczfalusy et al., 2011).

CRP was measured by a commercial kit, Tina-quant C-reactive Protein Gen.3, Roche Diagnostics GmbH, Mannheim, Germany run on a Modular P EVO, Roche Diagnostics GmbH, Mannheim, Germany.

Statistical analysis

Statistical analyses were performed using GraphPad Prism software version 5.03 (San Diego, CA, USA) and R 2.11.1. Correlations were studied using the non-parametric Spearman's test and confidence intervals were calculated using the adjusted bootstrap percentile (BCa) method with 1000 resamplings. 4β-hydroxycholesterol levels were compared between men and women using Student's t-test (two-tailed, unpaired). The modulating effects of specific genotypes on the association between plasma 25-hydroxyvitamin D and 4β-hydroxycholesterol were investigated by means of linear regressions. In each regression, log-transformed 4β-hydroxycholesterol values were regressed on 25-hydroxyvitamin D concentrations, genotypes (Bergman et al., 2012) and a 25-hydroxyvitamin D x genotype interaction term. Values of $p < 0.05$ were considered to be statistically significant.

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Results

Correlation between 25-hydroxyvitamin D and 4β-hydroxycholesterol levels

The 25-hydroxyvitamin D levels in this cohort spanned from 10 to 284 nmol/ L and the 4β-hydroxycholesterol levels ranged from 14.2 to 94.4 ng/mL. There was no correlation between individual 25-hydroxyvitamin D levels and the 4β-hydroxycholesterol levels (Spearman's r 0,002; 95% confidence interval -0.192 to 0.201), (Figure 1A). There was no correlation in a sub-groups analysis when the vitamin D-treated patients (25-hydroxyvitamin D levels from 65-284 nmol/L) and the placebo-treated patients (25-hydroxyvitamin D levels 10-114 nmol / L) were analysed separately (Figures 1B and C). The 4β-hydroxycholesterol levels were lower in men than in women, mean values 31.8 ng/mL compared to 39.1 ng/mL ($p<0.05$, two-tailed t-test), in accordance with earlier reports (Diczfalusy et al., 2008). Even when women and men were analysed separately there was no correlation between 25-hydroxyvitamin D levels and 4β-hydroxycholesterol (Figures 1D and E). Since many drugs may affect CYP3A4 expression and activity, the medical record for each patient was carefully assessed. No drugs with a major impact on CYP3A4 activity (according to the Flockhart Drug Interaction list; http://medicine.iupui.edu/clinpharm/ddis/table.aspx) were found, except carbamazepine and this single patient was already excluded from the study. However, 11 patients were prescribed oral glucocorticoids in low doses, which theoretically could induce CYP3A4 activity (Matsunaga et al., 2012). Thus, a separate analysis without these patients' data was performed and there was still no correlation between 25-hydroxyvitamin D and 4β-hydroxycholesterol levels (data not shown).

Vitamin D gene polymorphisms and 4β-hydroxycholesterol

Allelic variants of the SNPs Taq1 and Foq1 in the VDR gene had no influence on the association between 25-hydroxyvitamin D and 4β-hydroxycholesterol.

Correlation between CRP and 4β-hydroxycholesterol levels

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The CRP-values in the study cohort ranged from 0.2 to 73.8 mg/L (median 1.6 mg/L). There was a statistically significant negative correlation (Spearman's r -0.234; 95% c.i. -0.402 to -0.046) between CRP and 4β-hydroxycholesterol (Figure 2).

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Discussion

The aim of this study was to investigate whether the individual vitamin D-level correlates with CYP3A activity and therefore could be of predictive and therapeutic relevance for hepatic drug-metabolising capacity. The patient cohort under study, with marked variation in 25-hydroxyvitamin D levels (10-284 nmol/ L) provided a unique opportunity to study such an association with focus on the endogenous CYP3A4 biomarker 4β-hydroxycholesterol. Importantly, we did not detect any correlation between serum 25-hydroxyvitamin D levels and 4β-hydroxycholesterol. Much of the original work on this biomarker describes intra-individual changes in response to classical CYP3A4-inducers, such as anticonvulsant drugs and rifampicin (Bodin et al., 2001; Kanebratt et al., 2008). Therefore, it would have been of relevance to investigate possible changes in 4β-hydroxycholesterol over time from baseline in patients randomised to high-dose vitamin D. Unfortunately, these baseline serum samples were no longer available.

We previously demonstrated a seasonal variation in concentration-to-dose ratios for the CYP3A4-substrates tacrolimus and sirolimus, and proposed that this might be explained by seasonal changes in vitamin D. Seasonal variability in CYP3A4-activity was recently confirmed by independent researchers who studied CYP3A4 expression in the intestinal mucosa (Thirumaran et al., 2012). Here, the median CYP3A4 mRNA levels were more than three times higher during the months with higher sun-light exposure (April-September) than during the darker months (October-March). In another study, a daily dose of vitamin D (800 IU) for six weeks in 16 healthy subjects caused a significant reduction in the serum levels of atorvastatin, known to be metabolised by CYP3A4 (Schwartz, 2009). Combined, these results are compatible with a role of vitamin D in regulation of CYP3A4 gene expression, but perhaps the action of vitamin D on CYP3A4 gene expression is more specific for the intestinal mucosa. Interestingly in this respect, it has been shown that regulation of hepatic and intestinal CYP3A4 appears to be tissue-specific and independent from one another as further discussed below (von Richter et al., 2004).

4β-hydroxycholesterol is formed by CYP3A4 and CYP3A5. It has a long elimination time with a half-life of 17 days, resulting in stable circulating concentrations (Diczfalusy et al., 2009). The long half-

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life is an advantage during measurements under steady state conditions, but makes this marker less appropriate for studies on rapid changes in CYP3A activity, for instance during exposure to potent catalytic inhibitors. In contrast, 4β-hydroxycholesterol has been used extensively in studies on CYP3A induction (Kanebratt et al., 2008; Wide et al., 2008; Habtewold et al., 2012), showing good concordance with the response in other markers of CYP3A activity such as quinine metabolic ratio (Kanebratt et al., 2008) or the 6β-hydroxycortisol to cortisol ratio in urine (Mårde Arrhen et al., 2012). CYP3A4 expression is regulated by a complex network of transcription factors, e.g. the vitamin D receptor (VDR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Drocourt et al., 2002). It has been reported that long-term treatment of humans with the antiviral drug efavirenz led to induction of CAR target genes in the liver but not in the intestine (Meyer zu Schwabedissen et al., 2012). It has also been reported that PXR expression in humans is higher in the liver than in the intestine, whereas VDR expression is significantly higher in the ileum than in the liver (Khan et al., 2009). Since we did not see any effect of vitamin D supplementation on 4β-hydroxycholesterol formation, it is possible that 4β-hydroxycholesterol principally acts as a biomarker of hepatic CYP3A regulated by PXR/CAR and that the vitamin D receptor pathway has a limited influence on its formation.

Cell and animal experiments have shown that pro-inflammatory cytokines suppress CYP3A4-activity (Morgan et al., 2008; Morgan, 2009). In a small (n=26) recent study in patients with end-stage renal disease, an association between inflammation (increased CRP) and low CYP3A4 activity assessed by alprazolam 4-hydroxylation was noted, but there was no correlation between inflammation and 4β-hydroxycholesterol (Molanaei et al., 2012). Alprazolam 4-hydroxylation is a marker for rapid changes in CYP3A4-activity while 4β-hydroxycholesterol reflects CYP3A4 activity over longer time-periods and is more useful in determining induction than inhibition of CYP3A4. The study by Molanaei, including only 26 subjects, probably lacked the power necessary to detect an inhibition of CYP3A using 4β-hydroxycholesterol as a marker. In the present study cohort, being more than 4 times larger (n=116), we could show a statistically significant negative correlation between 4β-hydroxycholesterol and CRP. A causal link between inflammation and reduced hepatic CYP3A4 would be of potentially great clinical relevance and this research area warrants further studies on the kinetics of specific drugs in variable states of inflammation as well as during co-treatment with anti-inflammatory agents.

In summary, in the present cohort of patients with highly variable levels of 25-hydroxyvitamin D, we failed to see any correlation with the endogenous marker of CYP3A, 4β-hydroxycholesterol. Although the width of the confidence interval was such that a weak correlation ($r \leq 0.3$) cannot be ruled out, this suggests that individual vitamin D level in serum is not an important predictor of hepatic drug metabolism of CYP3A4-substrates. It remains to be understood if the previously observed seasonal variability in blood levels of CYP3A4 drugs substrates (Lindh et al., 2011) primarily reflects variability in intestinal first-pass metabolism of orally administered drugs. An important observation in the present study, however, was the negative relationship between individual CRP levels and the CYP3A4 biomarker 4β-hydroxycholesterol, which supports previous data that acute and / or chronic inflammation can suppress CYP gene expression in patients.

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Authorship contribution in alphabetical order:

Participated in research design: HN, LBB, PB, UD

Conducted experiments: ACN, HN, JDL, LBB, LE, PB, UD

Performed data analysis: EE, HN, JDL, LBB, LE, PB, UD

Wrote or contributed to the writing of the manuscript: EE, HN, JDL, LBB, LE, PB, UD

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Footnote:

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† LBB and HN contributed equally to this study and share first authorship.

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Legends to figures

Fig 1: Correlation between 25-hydroxyvitamin D-levels and 4β-hydroxycholesterol (4b-OH chol) in A) all 160 subjects in the study B) 58 subjects treated with 4000 IE/day for 6 months C) 58 subjects treated with placebo for 6 months D) The 85 women in the study; 44 receiving vitamin D and 41 placebo E) the 31 men in the study; 14 receiving vitamin D and 17 placebo. No significant correlations were found between 25-hydroxyvitamin D-levels and 4β-hydroxycholesterol in any of the analyses using Spearman's-test. There were significantly lower 4β-hydroxycholesterol levels in men than in women ($p<0.05$).

Fig 2: Correlation-study between 4β-hydroxycholesterol and CRP in 116 patients from the vitamin D study. A significant correlation was found between 4β-hydroxycholesterol (4b-OH chol) and CRP (Spearman's r -0.234; 95% c.i. -0.402 to -0.046).

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Fig. 1

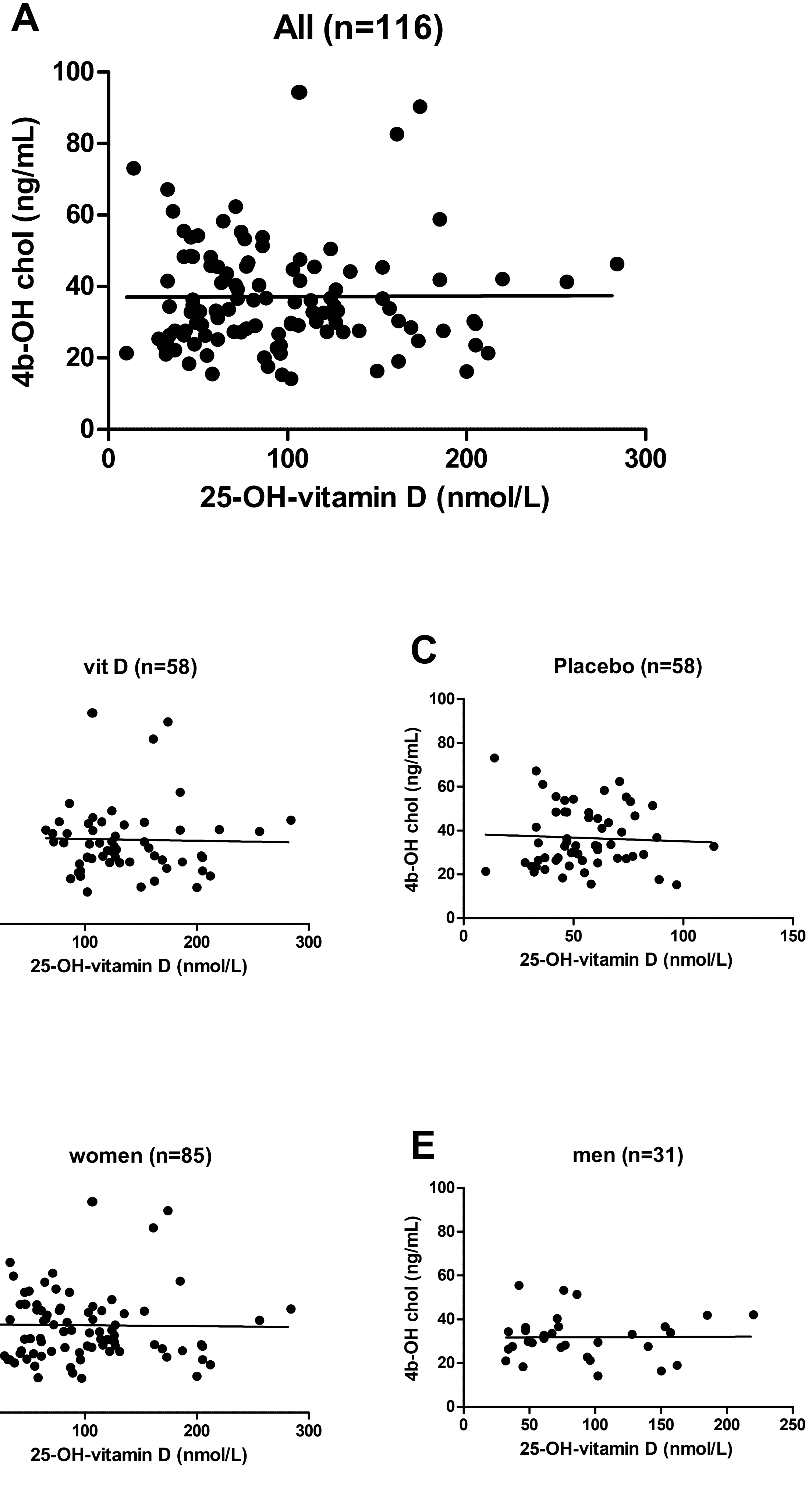

Fig. 2