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Platelet Integrin αIIbβ3 Activation is Associated with 25-Hydroxyvitamin D Concentrations in Healthy Adults

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Abstract

Background Cardiovascular events are associated with low circulating vitamin D concentrations, although the underlying mechanisms are poorly understood. This study investigated associations between 25-hydroxyvitamin D concentrations, platelet function, and single-nucleotide polymorphisms (SNPs) in genes influencing vitamin D biology in the 500 Functional Genomics (500FG) cohort.

Methods In this observational study, platelet activation and function were measured by flow cytometry by binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 and expression of P-selectin, markers of platelet aggregation and degranulation, respectively. These parameters were correlated to serum 25-hydroxyvitamin D and genotyping was performed to investigate SNPs in genes important for vitamin D biology.

Results Circulating 25-hydroxyvitamin D concentrations correlated inversely with baseline platelet binding of fibrinogen to integrin αIIbβ3 (Pearson's $r = -0.172$, $p = 0.002$) and platelet responses to platelet agonist cross-linked collagen-related peptide (CRP-XL) (Pearson's $r = -0.196$, $p = 0.002$). This effect was due to circulating vitamin D levels $\leq$50nmol/L, since no differences in platelet fibrinogen binding were observed between subjects with normal 25-hydroxyvitamin D concentrations (>75-nmol/L) and a 25-hydroxyvitamin D insufficiency (50–75 nmol/L). No correlations between 25-hydroxyvitamin D concentrations and platelet P-selectin expression were found. Several SNPs in the GC region of the vitamin D binding proteingene were associated with platelet responses to CRP-XL.

Keywords
- platelets
- vitamin D
- inflammation
- hemostasis
- fibrinogen

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Conclusion Low circulating vitamin D concentrations are associated with increased platelet fibrinogen binding to integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL. These findings may contribute to the increased incidence of cardiovascular events in vitamin D deficient adults and its seasonal variation. Further studies are needed to investigate causality.

Introduction

Cardiovascular diseases, such as myocardial infarction, are the leading causes of global mortality and morbidity.[1] A seasonal variation of its incidence is observed, with a higher rate of events in the winter and a nadir in the summer.[2–5] Vitamin D concentrations show a similar seasonal pattern as vitamin D is influenced by skin exposure to sunlight.[6] Many studies have shown associations between low vitamin D concentrations and cardiovascular diseases[7–10] and the majority of patients with acute myocardial infarction are vitamin D deficient.[11,12] However, the underlying mechanisms remain poorly understood.

Vitamin D has many functions beyond its traditional role in bone health, including a regulatory role in inflammation and infection.[13–15] Inflammation and hemostasis are closely linked biological systems,[16] and during episodes of increased systemic inflammation, thrombotic complications are more frequently observed.[17] Platelets play a pivotal role in this process and platelet activation, degranulation, and aggregation are essential steps in arterial thrombus formation.[18] Interestingly, it has been reported that both platelets and their megakaryocyte precursors express the vitamin D receptor (VDR).[19] However, the direct association between vitamin D and platelet function in humans is poorly studied.

The vitamin D pathway is a complex metabolic pathway that has many steps before the substrate 25-hydroxyvitamin D is converted in its active metabolite 1,25-dihydroxyvitamin D.[6,13] As a result, there is a large variety of genetic interindividual factors that influence vitamin D homeostasis.[10,20,21] Single-nucleotide polymorphisms (SNPs) in genes of several key factors influencing vitamin D biology, such as vitamin D binding protein (VDBP), cytochrome P2R1 (CYP2R1), and VDR, are reported to influence 25-hydroxyvitamin D concentrations, but may also influence 1,25-dihydroxyvitamin D bio-availability and thereby its physiological effects.[20–24]

For this study, data from the 500 Human Functional Genomics Project (500FG) were used. The 500FG is part of the Human Functional Genomics Project (HFGP) that aimed to characterize variations of immune cell function and platelet function in healthy adults.[25,26] Our group previously reported on associations between platelet number and reactivity with inflammation in the same 500FG study cohort.[26] Here, we report on associations of 25-hydroxyvitamin D concentrations, as well as genetic variation in genes involved in vitamin D biology, with platelet reactivity.

Methods

Study Design and Population

The 500FG cohort consists of 534 healthy volunteers and is part of the HFGP (http://www.humanfunctionalgenomics.org/site/) aimed at characterizing variations in immune function.[25] The study design and population have been previously described,[25,26] and the 500FG study was approved by the Ethical Committee of the Radboud University Medical Center (NL42561.091.12, 2012/550). In summary, between August 2013 and December 2014 a total of 534 healthy human subjects of Caucasian origin were recruited in the RadboudUniversity Medical Center, Nijmegen, the Netherlands. Participants were scheduled for a study visit between 8 and 10 a.m. to donate blood. After their visit, participants received an online questionnaire on dietary habits, lifestyle, and disease history.

Ethics

This study was approved by the local Ethical Committee (NL42561.091.12, 2012/550) and was conducted according to the principles of the Declaration of Helsinki (version October 2008) and in accordance with the Dutch Medical Research involving Human Subjects Act. All participants gave written informed consent before blood was drawn.

Blood Sampling and 25-Hydroxyvitamin D Measurement

Blood was drawn in sterile ethylenediaminetetraacetic acid, serum, and 3.2% sodium citrate Vacutainertubes (Becton Dickinson, Plymouth, United Kingdom). 25-Hydroxyvitamin D3 was measured with liquid chromatography-tandem mass spectrometry after precipitation of the protein and solid-phase extraction as described in further detail by ter Horst et al.[25] In summary, an internal standard of ^{2}H3 25OH-vitamin D3 was added before 50 µLNaOH (2M) was added to release protein-bound 25-OH vitamin D3 and a combination of acetonitrile/methanol (9:1) was added for protein precipitation. H_2O was added followed by solid-phase extraction (Oasis HLB 1cc, Waters, Etten-Leur, the Netherlands). The eluate (300 µL methanol/isopropanol 95:5) was diluted with H_2O (3:1) and injected (10 µL) into an Agilent Technologies 1290 Infinity VL UHPLCsystem (Agilent Technologies, Santa Clara, California, United States), equipped with a BEH C18 (1.7 µm 2.1 × 50mm) analytical column (Waters) at 45°C. An Agilent 6490 tandem mass spectrometer (Agilent Technologies) was operated in the electrospray positive ion mode, with a capillary voltage of 3.5 kV, fragmentor voltage of 380 V, sheath gas temperature of 350°C, and gas temperature of 100°C with N_2 collision gas. Both 25OH-vitamin D3 and 25OH-vitamin D3

[-H2O] (in-source fragmentation) were used for quantification (results were averaged) with both two transitions (qualitative and quantitative) monitored. An 8-point calibration curve was used and absolute concentration of the calibrator (Sigma-Aldrich, Zwijndrecht, the Netherlands) was assessed by spectrophotometry (264 nm). The linearity of the method was assessed by the Clinical and Laboratory Standards Institute EP6 protocol and recovery rates were within 90 to 109%.

Platelet Activation and Function Assessment

Platelet activation was defined by the binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 (glycoprotein [GP] IIb/IIIa complex) and the expression of P-selectin (CD62P) on the platelet surface, markers of platelet aggregation and degranulation, respectively. Platelet activation was measured in whole blood samples at baseline and after incubation with different platelet agonists, to assess its functional capacity. The agonists used were adenosine 5′ diphosphate (ADP) (Sigma-Aldrich, Saint Louis, Missouri, United States) and cross-linked collagen-related peptide (CRP-XL) (kind gift from Prof. Dr. R. Farndale, Cambridge, United Kingdom). The blood samples were incubated for 20 minutes with 8 different concentrations of the agonists in combination with antibodies for flow cytometry at room temperature, followed by fixation with 0.2% paraformaldehyde. Staining of samples was performed with antibodies for CD61 (PC7-conjugated) (Beckman Coulter Brea, California, United States), anti-human fibrinogen (fluorescein isothiocyanate-conjugated) (Dako), and P-selectin (CD62P, phycoerythrin-conjugated) (Biolegend, San Diego, California, United States). The expression of these markers was measured by flow cytometry (FC500 Flow Cytometer, Beckman Coulter). Gating of platelets was performed based on forward and sideward scatter and additionally for CD61 positivity. The area under the curve of fibrinogen binding and P-selectin expression after stimulation (median fluorescence intensity) was used for correlations with 25-hydroxyvitamin D concentrations and SNPs.

Genotyping

The deoxyribonucleic acid samples of the participants were genotyped with a commercially available SNP chip, Illumina HumanOmniExpressExome-8 v.1.0, methods previously reported by Li et al.[27] In short, genotype calling was performed using Optical 0.7.0. Call rates less than ≤0.99 were excluded from the dataset, as were samples with a Hardy–Weinberg equilibrium ≤0.0001, call rate ≤0.99, and minor allele frequency ≤0.001. A total of 483 samples were left for the genetic analysis, as described previously.[27] Of the 39 SNPs involved in the vitamin D pathway that were identified from literature,[10,28–30] 31 SNPs were available in our dataset, that is, rs10741657, rs10877012, rs2134095, rs2282679, rs3829251, rs10766197, rs218174, rs1155563, rs12785878, rs12794714, rs2762933, rs7041, rs6599638, rs10500804, rs7975232, rs4588, rs6055987, rs7116978, rs3755967, rs12800438, rs1562902, rs17467825, rs3794060, rs1993116, rs7968585, rs4945008, rs2060793, rs222020, rs4944957, rs2298849, and rs1801222.

Statistical Analyses

Statistical analysis was performed with IBM SPSS statistics 22.0 (New York, New York, United States), R (Vienna, Austria), and Graphpad Prism 5.0 (San Diego, California, United States). All data were tested for normality with the Shapiro–Wilkinson test and assessed in corresponding Q-Q plots. Nonnormally distributed data were log-transformed before further analyses. Pearson's *R* correlation coefficients were calculated in R using the standard cor.test.routine. The nominal *p*-value of <0.05 was used as the significance threshold. Correction for multiple comparisons was applied using false discovery rate.

Results

The demographics of the study population are presented in ►**Table 1**. Most participants were in their early adulthood and had a normal body mass index. The Netherlands has a strong annual variation in terms of sunlight exposure, and as participants were included throughout the year, we found an absolute vitamin D deficiency (≤ 50nmol/L) in 105 participants. The variation in vitamin D concentrations during recruitment was previously published by ter Horst et al.[25]

Low 25-Hydroxyvitamin D3 Concentrations Correlate to Platelet Binding of Fibrinogen to Integrin αIIbβ3

A modest, but statistically significant inverse correlation was observed between 25-hydroxyvitamin D concentrations and platelet binding of fibrinogen to the activated fibrinogen receptor integrin αIIbβ3 in unstimulated samples (Pearson's $r = -0.172$, $p = 0.002$, $n = 393$) (►**Fig. 1A**). Platelet fibrinogen

Table 1 Demographics of study participants

		25-Hydroxyvitamin D concentrations			
Characteristics	Total	≤ 50 nmol/L	50–75 nmol/L	>75 nmol/L	*p*-Value
n (% of whole cohort)	486 (100)	106 (19.7)	174 (32.6)	206 (38.6)	–
Gender (% male)	43.3	62.9	44.3	32.5	<0.001
Mean age, y (SD)	27.1 (12.9)	26.6 (13.3)	27.2 (12.5)	27.3 (13.0)	ns
BMI (SD)	22.7 (2.7)	23.0 (3.2)	22.7 (2.6)	22.4 (2.5)	ns
Smoker (%)	13.1	10.5	11.5	13.1	ns
OC use (% of women)	53.0	–	–	–	–

Abbreviations: BMI, body mass index; ns, nonsignificant at the $p < 0.05$ level; OC, oral contraceptive; SD, standard deviation.

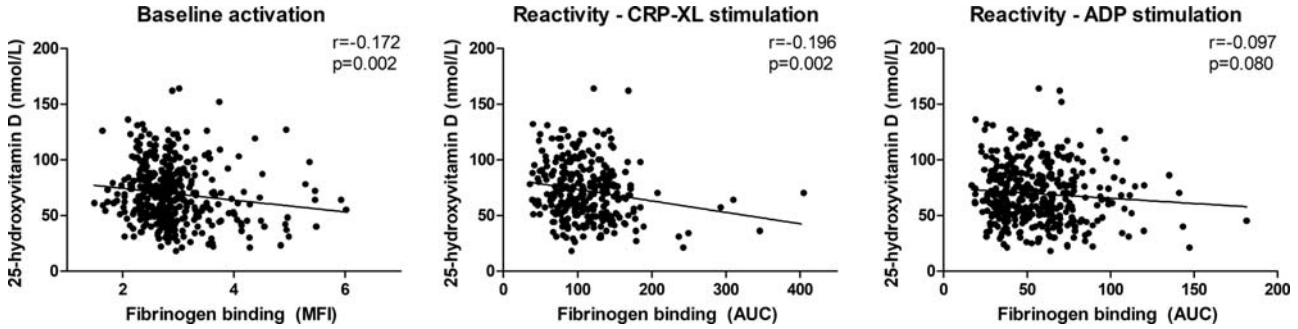

Fig. 1 Correlation between vitamin D and platelet fibrinogen binding. The correlation between 25-hydroxyvitamin D and platelet fibrinogen binding in (**A**) unstimulated samples (fibrinogen MFI) and in response to stimulation with platelet agonists (**B**) CRP-XL and (**C**) ADP (both expressed as AUC of fibrinogen MFI). ADP, adenosine diphosphate; AUC, area under the curve; CRP-XL,cross-linked collagen-related peptide; MFI, median fluorescence intensity.

binding in response to platelet stimulation of GPVI-receptor by CRP-XL also inversely correlated to vitamin D concentrations (Pearson's $r = -0.196, p = 0.002, n = 299$) (►**Fig. 1B**). These data show higher platelet activation and reactivity in participants with low vitamin D concentrations. No correlation between vitamin D levels and platelet reactivity in response to stimulation with platelet agonist ADP (Pearson's $r = -0.097$, $p = 0.08$, $n = 393$) was observed (►**Fig. 1C**). Interestingly, P-selectin expression, a marker of platelet degranulation, was not affected by vitamin D concentrations in unstimulated samples (Pearson's $r = 0.037$, $p = 0.52$, $n = 467$) (►**Fig. 2A**), nor was there a correlation in stimulated CRP-XL and ADP stimulated samples (Pearson's $r = -0.12$, $p = 0.08$, $n = 299$ and Pearson's $r = 0.02$, $p = 0.66$, $n = 486$, respectively) (►**Fig. 2B, C**).

25-Hydroxyvitamin D Deficiency (≤50 nmol/L), but not Insufficiency, Seems to be Correlated to Platelet Integrin αIIbβ3 Activation

Next, study participants were grouped according to the commonly used cut-off values for 25-hydroxyvitamin D concentrations; subjects with vitamin D concentrations >75 nmol/L were considered sufficient, subjects with vitamin D concentrations from 50 to 75 nmol/L were considered insufficient, and the threshold for vitamin D deficiency was ≤ 50 nmol/L. A significant increase was found in platelet fibrinogen binding to integrin αIIbβ3 in unstimulated samples and in response to stimulation with CRP-XL in vitamin D deficient participants compared with sufficient and insufficient participants (►**Fig. 3**). Importantly, no significant differences were observed between vitamin D sufficient and vitamin D insufficient participants in terms of fibrinogen binding, nor for P-selectin expression between any of these three groups.

When applying the commonly used conservative threshold of 50 nmol/L, fibrinogen binding in unstimulated samples was significantly ($p < 0.001$) higher in participants with vitamin D ≤50 nmol/L (mean 0.49 ± 0.009) compared with participants with vitamin D >50 nmol/L (mean 0.44 ± 0.005). This difference in fibrinogen binding was also observed after stimulation with CRP-XL (vitamin D ≤50 nmol/L: mean 2.088 ± 0.02 vs. vitamin D >50 nmol/L: mean 1.997 ± 0.01, $p < 0.001$) and after stimulation with ADP (vitamin D ≤50 nmol/L: mean 1.744 ± 0.02 vs.vitamin D >50 nmol/L: mean 1.696 ± 0.01, $p = 0.021$)(►**Supplementary Fig. S1**, available in the online version). Together, these data suggest that platelet fibrinogen binding to integrin αIIbβ3 is stronger in vitamin D deficient participants.

Interestingly, P-selectin expression after CRP-XL stimulation was significantly ($p = 0.020$) higher in participants with vitamin D ≤ 50 nmol/L (mean 301 ± 9) compared with participants with vitamin D >50 nmol/L (mean 269 ± 4), whereas this was not observed in unstimulated samples (vitamin D >50 nmol/L: mean 0.71 ± 0.004 vs.vitamin D ≤ 50 nmol/L:

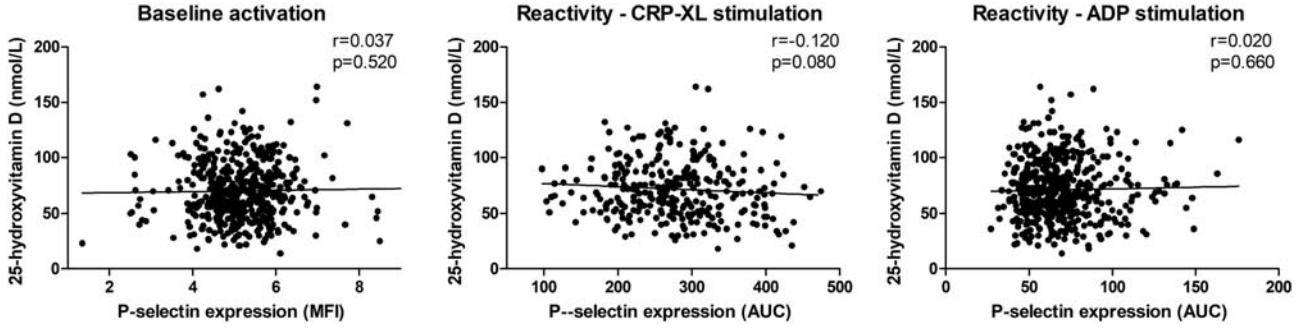

Fig. 2 Correlation between vitamin D and platelet P-selectin expression. The correlation between 25-hydroxyvitamin D and platelet P-selectin expression in (**A**) unstimulated samples (P-selectin MFI), and in response to stimulation with platelet agonist (**B**) CRP-XL and (**C**) ADP (both expressed as AUC of P-selectin MFI). ADP, adenosine diphosphate;AUC,area under the curve; CRP-XL,cross-linked collagen-related peptide; MFI, median fluorescence intensity.

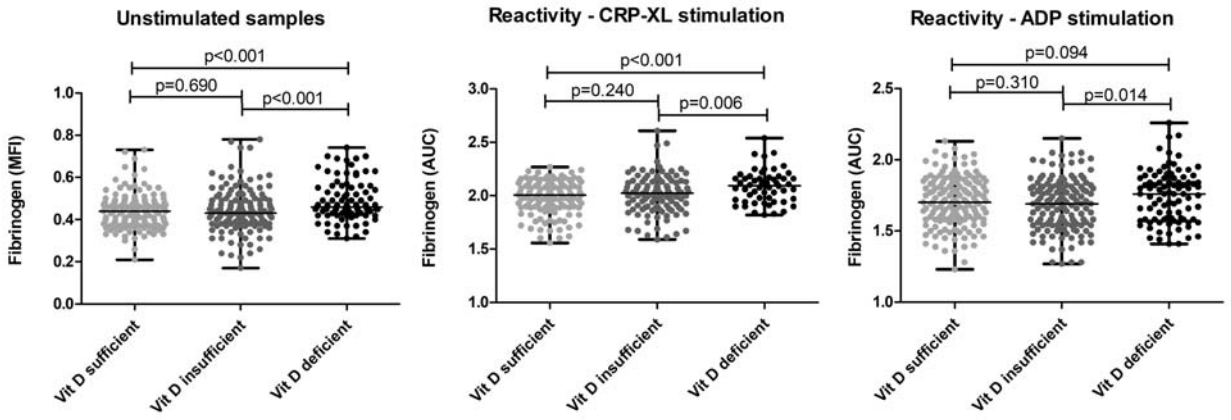

Fig. 3 Vitamin D status and platelet fibrinogen binding.Fibrinogen binding (**A**) at baseline and after stimulation with (**B**) CRP-XL or (**C**) ADP in vitamin D sufficient (>75 nmol/L), insufficient (50–75 nmol/L), and deficient (≤ 50 nmol/L) participants. ADP, adenosine 5′diphosphate; AUC, area under the curve; CRP-XL, cross-linked collagen-related peptide; MFI, median fluorescence intensity; Vit D, 25-hydroxyvitamin D.

mean 0.70 ± 0.010, $p = 0.320$), nor after stimulation with ADP (vitamin D >50 nmol/L:mean 1.82 ± 0.01 vs.vitamin D ≤50 nmol/L: mean 1.83 ± 0.01, $p = 0.580$) (►**Supplementary Fig. S2**, available in the online version).

The Influence of SNPs in Key Players of the Vitamin D Pathway on Platelet Activation and Function

The vitamin D pathway is a complex metabolic pathway that is regulated on many levels. SNPs in genes encoding for key proteins in this pathway, such as *VDBP*, *CYP2R1*, and *VDR*, were determined and related to platelet activation and platelet function. Thirty-nine functional SNPs in the vitamin D pathway were identified through a thorough literature search, of which 31 SNPs were available in the dataset. In total, 9 SNPs had a significant influence on fibrinogen binding to integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL or ADP (►**Table 2**). Six out of 9 associated SNPs were present in the GC region of *VDBP*, of which 5 were associated with platelet fibrinogen binding to integrin αIIbβ3 in response to stimulation with CRP-XL, indicating that there may be crosstalk in these signaling pathways. Next, we examined whether these SNPs exerted their effects on platelet activation and function through vitamin D concentrations or in an independent manner by performing causality tests.[31,32] The analyses on each SNP–vitamin D platelet triple result in an "independent" model, suggesting that the genetic effect on both phenotypes are statistically independent, or there is a limited power to detect the actual causal relationship using the dataset available.

Discussion

This is the first study that investigated the correlation between 25-hydroxyvitamin D concentrations and platelet activation

Table 2 Relation between fibrinogen binding to αIIbβ3 and SNPs in the vitamin D pathway

SNP	rs10877012	rs6599638	rs2282679	rs1155563	rs7041	rs4588	rs3755967	rs17467825	rs1801222
Gene	*CYP27B1*	*C10orf88*[a]	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*GC (VDBP)*	*CUBN*[b]
Alleles	G/T	G/A	T/G	T/C	A/C	G/T	C/T	A/G	G/A
Minor allele	T	A	G	C	A	T	T	G	A
Minor allele effect	Decreasing	Increasing	Decreasing	Decreasing	Increasing	Decreasing	Decreasing	Decreasing	Increasing
MAF	0.35	0.43	0.27	0.30	0.59	0.27	0.27	0.27	0.63
Unstimulated	0.036	ns	ns	ns	ns	ns	ns	ns	ns
Reactivity, CRP-XL	ns	ns	0.05	0.03	ns	0.02	0.04	0.05	0.05
Reactivity, ADP	ns	0.004	ns	ns	ns	ns	ns	ns	ns

Abbreviations: ADP, adenosine 5′diphosphate; CRP-XL, cross-linked collagen-related peptide; CUBN, cubilin; MAF, minor allele frequency; orf, open-reading frame; SNP, single-nucleotide polymorphism; VDBP, vitamin D binding protein.
Note: The *p*-values of the association between fibrinogen binding in unstimulated samples and samples stimulated with CRP-XL or ADP and the different SNPs in the vitamin D pathway are displayed. The raw *p*-values are listed in the table.
[a]The region harboring the open-reading frame 88 (C10orf88) on chromosome 10q26.13.
[b]Cubilin is important for vitamin D uptake into cells by binding vitamin D to VDBP.

and reactivity in a large cohort of healthy Caucasian adults. We found an inverse correlation between 25-hydroxyvitamin D concentrations and fibrinogen binding to the activated platelet fibrinogen receptor integrin αIIbβ3 in unstimulated samples and after stimulation with the platelet agonist CRP-XL. This indicates increased platelet activation and reactivity in individuals with low vitamin D concentrations. When 25-hydroxyvitamin D concentrations $\leq$50 nmol/L was used as a cut-off value, fibrinogen binding to integrin αIIbβ3 in response to agonist ADP was also significantly associated. No associations between platelet function parameters and 25-hydroxyvitamin D insufficiency and normal 25-hydroxyvitamin D concentrations were observed, further supporting the presence of a threshold. Several SNPs in key genes contributing to the vitamin D pathway were significantly associated with platelet fibrinogen binding to integrin αIIbβ3 at baseline and/or in response to stimulation. Five out of 9 SNPs in the GC region of *VDBP* were associated with fibrinogen binding in response to CRP-XL. Several associations between the SNPs, 25-hydroxyvitamin D, and platelet function parameters were found, however, causality could not be formally confirmed.

We report a modest, but significant effect of vitamin D concentrations on platelet fibrinogen binding to the activated fibrinogen receptor integrin αIIbβ3. However, on a population level, small effects may have considerable impact. Myocardial infarction and stroke are the leading causes of death worldwide with 8 million deaths a year.[33] Cardiovascular diseases are multifactorial in origin and a combination of factors, such as atherosclerosis, plaque instability, and platelet reactivity, contribute to its development.[34] Associations with vitamin D concentrations exist, although these are poorly understood.[7]

Previously, few studies investigated direct associations between vitamin D concentrations and platelet parameters in health and disease.[35–40] Verdoia et al reported an association between low vitamin D concentrations and high-residual platelet reactivity in patients with cardiovascular diseases receiving dual antiplatelet therapy,[35] and similarly this was shown in patients with diabetes.[38] Moreover, in hemodialysis patients vitamin D supplementation resulted in antithrombotic effects.[37] These findings, together with our observations, may point out specific pathways that are functionally involved.

The strongest association with 25-hydroxyvitamin D was found for platelet fibrinogen binding in response to CRP-XL stimulation. Interestingly, 5 of the investigated SNPs in the GC region, coding for VDBP, also showed an association with platelet responses to CRP-XL. The platelet agonist CRP-XL signals through the collagen pathway to activate platelets and is dependent on phosphorylation of signal transducer and activator of transcription 3 (STAT3).[41] Interactions between vitamin D and JAK-STAT signaling pathways have been reported.[42,43] Olson et al showed that vitamin D decreases STAT3 phosphorylation, and this may be a mechanistic explanation for the increased platelet fibrinogen binding in response to CRP-XL in vitamin D deficient participants.[43] VDBP itself may also play a key role, being the major vitamin D transporter in the circulation, and thus an important determinant of the circulating active vitamin D pool and its biological effect. In support of this, López-Farré et al reported that increased VDBP levels are associated with reduced inhibitory effects of aspirin on platelet adhesion.[44] Moreover, Verdoia et al observed that vitamin D deficient carriers of the G allele of the VDBP rs7041 polymorphism had high residual platelet reactivity in response to ADP despite dual antiplatelet therapy.[45] These findings suggest that VDBP could prevent binding of vitamin D to its receptors on platelets and thereby modulate the (antithrombotic) platelet response to various agonists.

In contrast to platelet fibrinogen binding, platelet P-selectin expression was only associated with 25-hydroxyvitamin D in response to stimulation with CRP-XL in vitamin D deficient adults ($<$50 nmol/L). Moreover, there was no association between P-selectin expression upon CRP-XL stimulation and any of the SNPs. The involvement of P-selectin seems far less than fibrinogen, and it is likely that the study is underpowered to detect such small effects. Others have suggested that platelet functional responses may be differentially regulated, which may be a plausible explanation for the different observations for fibrinogen binding and P-selectin expression.[46]

A causal relationship between the SNPs studied here, vitamin D concentrations, and platelet function parameters could not be confirmed, and caution is warranted as it has been suggested that vitamin D concentrations are merely a reflection of overall health status. People with a low health status may have a more sedentary lifestyle, may stay more indoors, and, in combination with a poor dietary variation, this results in low vitamin D concentrations. Therefore, vitamin D could be an epiphenomenon and may not be causally involved. This is further strengthened by supplementation studies that yield little to no effect on cardiovascular diseases to date.[47–49]

To increase our understanding of the involvement of vitamin D in hemostasis, future studies should focus on the underlying pathways. A combination of many factors ultimately determines disease susceptibility and progression. It may be worthwhile to investigate the effects of vitamin D supplementation on specific factors such as platelet reactivity. An example is the PRECOVID trial, which investigates the effects of vitamin D supplementation on immune responses and platelet function in vitamin D deficient chronic obstructive pulmonary disease patients (first results expected end of 2020).[50] Furthermore, causal inference analyses should be pursued in large cohorts as it is pivotal to know whether vitamin D acts as a mediator or is just an epiphenomenon. Insight in the exact mechanisms is important to further understand the clinical consequences of vitamin D deficiency as well as to identify effective therapeutic opportunities.

Our study has a few limitations. One important limitation is the observational design of the study and this study must therefore be considered as a hypothesis-generating study. This cohort was relatively large and we tried to perform formal causal inference analyses; however, as the effect is relatively small, the study may be underpowered to detect these subtle differences and causality could not be shown. Second, there were significantly more men in the vitamin D deficient group. Our previous publication on platelet function in this cohort by Tunjungputri et al showed no significant associations between gender and platelet reactivity.[26]

Verdoia et al reported that female gender was associated with lower vitamin D levels.[51] However, this study included patients undergoing coronary angiography and may therefore have a different participant selection. Our last limitation is that many participants were relatively young and this may lead to an underestimation of the effects of vitamin D.

In conclusion, this study shows increased platelet fibrinogen binding to integrin αIIbβ3 in healthy adults with low vitamin D concentrations, particularly below 50 nmol/L. This observation may partially explain the association with thrombotic diseases and its seasonal variation. Further studies are needed to investigate the underlying mechanisms and causality of vitamin D in platelet function.

What is known about this topic?

- Low vitamin D concentrations have been associated with a higher risk of cardiovascular disease.
- Besides its traditional role in bone health, vitamin D has a regulatory role in inflammation and infection.
- Platelets play key roles in both inflammation and hemostasis, and express the vitamin D receptor.

What does this paper add?

- 25-hydroxyvitamin D concentrations correlate inversely with fibrinogen binding to the activated platelet fibrinogen receptor integrin αIIbβ3 in unstimulated samples and after stimulation with CRP-XL.
- Several single-nucleotide polymorphisms (SNPs) in the vitamin D binding protein (VDBP)gene were associated with platelet responses to CRP-XL.
- Our findings may partially explain the association between vitamin D deficiency and thrombotic diseases and its seasonal variation.

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Conflict of Interest
None declared.

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