Thrombin generation and fibrin clot structure after vitamin D supplementation

Short title: vitamin D and thrombin generation

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Abstract

Objective: Vitamin D deficiency is associated with increased risks of arterial and venous cardiovascular events. Hypothetically, supplementation with vitamin D may lead to a less prothrombotic phenotype, as measured by global coagulation assays and fibrin clot structure.

Methods: In this prospective cohort study, we enrolled adult outpatients attending the Primary Care division of the Geneva University Hospitals with a severe vitamin D deficiency (25-hydroxyvitamin-D3 (25-OHD) <25nmol/L), excluding obese patients or with a recent acute medical event. We evaluated changes in coagulation times, thrombin generation assay, clot formation and clot lysis time, 25-OHD and parathormone before and 1-3 months after cholecalciferol oral supplementation with one-time 300'000IU then 800UIIU daily. Paired t-tests with a two-sided alpha of 0.05 compared absolute mean differences.

Results: The 48 participants had a mean age of 43.8±13.8 years. After supplementation, 25-OHD levels increased from 17.9±4.6 nmol/L to 62.5±20.7 nmol/L 6.4+/-3.0 weeks after inclusion. Endogenous thrombin potential and thrombin generation peak values both decreased significantly (-95.4nM*min (95%CI -127.9 to -62.8), P<0.001; -15.1nM (-23.3 to -6.8), P<0.001). The maximum absorbance by turbidimetry decreased significantly (P=0.001) after supplementation. There was no change in clot lysis time, coagulation times or plasminogen activator inhibitor-1 and homocysteine levels.

Conclusions: In severe vitamin D deficiency, a high-dose cholecalciferol supplementation was associated with a reduction in thrombin generation and an average decreased number of fibrin protofibrils per fibers and fibrin fiber size

measured by turbidimetry. This suggests that severe vitamin D deficiency may be

associated with a potentially reversible prothrombotic profile.

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Introduction

Thrombosis, collectively including ischemic heart disease, stroke and venous thromboembolism (VTE), causes more than one in four deaths worldwide.[1] Venous thromboembolism itself is among the largest contributor of loss of disability-adjusted life-years in both high and low income countries. With this massive burden, prevention efforts may yield important positive effects on the population's health.

In observational studies, vitamin D deficiency is associated with increased future risks of arterial cardiovascular disease (CVD) and, perhaps, of VTE.[2-4] In contrast, vitamin D interventional studies have not demonstrated a protective role of cholecalciferol supplementation on cardiovascular health.[5-7] Given that such randomized studies were set among generally healthy adults, and with the expected healthier status of trial participants, severe vitamin D deficiency [as defined by 25-hydroxyvitamin (25-OHD) levels <25-30nmol/L] was under-represented in these trials. However, the high prevalence of severe vitamin D deficiency highlights its public health relevance, because about one in 7-8 European citizens suffer from this condition [8].

While several mechanisms linking vitamin D with CVD have been studied, whether vitamin D deficiency or supplementation affects hemostasis remains largely unexplored. Previous studies have reported cross-sectional associations between levels 25-OHD and tissue plasminogen activator (tPA), thrombin generation, plasminogen activator inhibitor-1 (PAI-1) and tissue factor pathway inhibitor (TFPI),[9-11] building on previous *in vitro* reports of a prothrombotic environment in vitamin D deficiency.[12,13] Three cohorts have studied the influence of vitamin D supplementation on thrombin generation, a global measure of hemostasis, and have led to contradictory results .[11, 14, 15] The fibrin clot structure, which has not been

studied in relationship to vitamin D, is a major determinant of the mechanical properties of the clot and its resistance to fibrinolysis, and appears altered in a variety of disease of thrombotic or hemorrhagic phenotypes.[16,17]

We hypothesized that supplementation with vitamin D in deficient patients would be associated with a less prothrombotic hemostatic profile, as measured by thrombin generation and fibrin clot structure, and tested it in a prospective cohort of outpatients with severe 25-OHD deficiency.

Material and Methods

This prospective cohort was set in the Primary Care Clinic of the Geneva University Hospitals, from 06.2016 to 06.2017. The study was approved by the Ethics Committee (Commission cantonale d'éthique de la recherche de Genève, 2016-00124), and informed consent was obtained from all participants after full explanation of the purpose and nature of all procedures used.

Population

We screened the results of all measurements of 25-OHD performed in adult outpatients. Vitamin D status is evaluated routinely in clinical practice at the first medical encounter and in follow-ups, when deemed necessary. Inclusion criteria were an age ≥18 years and severe vitamin D deficiency defined as a level of 25-OHD <25nmol/L (10ng/mL). Exclusion criteria were an ongoing treatment with anticoagulant or calcium/vitamin D supplements, an active cancer (diagnosed within 5 years or treated within 2 years), renal insufficiency (eGFR <30ml/min), a primary hyperparathyroidism, any malabsorption disease, an active inflammatory disease, a recent infection, surgery or hospitalization (<30 days), obesity (BMI>30kg/m^2), a

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recent thromboembolic or cardiovascular event (<90 days), or an ongoing

pregnancy.

Vitamin D supplementation

Following local recommendations for supplementation in patients with vitamin D

deficiency, all participants received a one-time high-dose oral cholecalciferol

(300'000IU) delivered directly at the Clinic, and a prescription for oral cholecalciferol

(800IU daily), without calcium supplement. This regimen had been recommended in

the context of poor adherence to daily or weekly low doses of vitamin D in this

population.

Data collection, variables and outcomes

Participants were evaluated at baseline, shortly after the diagnosis of vitamin D

deficiency, to collect data on demographics, medical history and a blood sample was

collected through a peripheral phlebotomy. One to three months after inclusion and

after the start of vitamin D supplementation, participants were invited for the 2nd

blood collection.

Most variables, including race, smoking, fractures, and menopause status, were self-

reported. The electronic medical chart, including all laboratory results, was checked

to evaluate the presence of co-morbidities and prescribed medications. Blood

pressure and BMI were measured at baseline.

The primary outcome of the study was the change in thrombin generation

biomarkers (endogenous thrombin potential, ETP) and fibrin clot structure

measurements (clot lysis time and maximal absorption by turbidimetry). As

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secondary outcomes, we also measured levels of PAI-1 and homocysteine, given previously reported cross-sectional associations with 25-OHD levels.[9]

Blood analyses

Non-fasting blood samples were collected into a tube containing 0.109 M citrate (BD Vacutainer, Plymouth, UK) after having discarded the first few milliliters. Blood was processed and centrifuged rapidly after collection, within at most 90 minutes. Routine analyses, including 25-OHD, calcium, PTH levels and coagulation times, were performed at the time of blood draw. All other assessments were performed batchwise by the same laboratory technician who was blind to the time of sampling (pre or post-supplementation), on plasma samples frozen on average for 48±24 days after a double centrifugation procedure (2000g for 10 min then 3900g for 10 min). Coagulation assays were performed on a BCS-XP (Siemens, Marburg, Germany) automate, using Pathromtin SL (Siemens) for the activated partial thromboplastin time (aPTT) and Innovin (Siemens) for the prothrombin time (PT). Plasma level of functional fibrinogen was measured by the Clauss method using Thrombin 100 IUI/ml (Siemens). 25-OHD levels and PTH were measured by an electro-chemiluminescence binding assay using the COBAS automate (Roche, Switzerland). Serum calcium was analyzed by photometrical assay (Roche).

Thrombin generation assay was performed with the calibrated automated thrombography (CAT) method (Stago, Asnières, France) by use of a flurometer (Fluroscan Ascent; ThermoLab Systems, USA) equipped with a dispenser.[18] Coagulation was initiated with 5 pM of tissue factor and 4 µM of phospholipids (PPP reagent, Stago). Calibration for each patient was performed with Thrombin Calibrator (Stago). All plates were incubated at 37° for 10 minutes before addition of the

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fluorogenic substrate and calcium chloride ($CaCl_2$; FluCa-Kit, Stago), and test were performed in triplicate. Raw data were analyzed with Thrombinoscope V5 (Stago). Pre- and post-supplementation fibrin polymerization and fibrinolysis were assessed by turbidity assays on plasma.[19] Plasma samples were diluted 1/6 with TBS (50 mM Tris, 100 mM NaCl, pH 7.4). Fibrin polymerization was monitored at 405 nm every 8 s for 120 min using a microplate reader (VersaMax, Molecular Devices, USA), after incubation with 0.1 U/ml human thrombin (Merck KGAa, Germany) and 5 mM $CaCl_2$ (final concentrations) in a final volume of 150 µl. Fibrinolysis (clot lysis time) was evaluated by adding 85 ng/ml of tPA (Technoclone GmbH, Austria) to aim at clot lysis time of about 20 minutes [20]. The inter-assay and intra-assay coefficients of variability were 4.7% and 5.4%, respectively The All measurements were made in triplicate and processed with the SoftMAX Pro software. Briefly, the maximum absorbance of the growing clot will reflect the average fibrin fiber size and the number of protofibrils per fiber. The clot lysis time corresponds to the time from the threshold of 50% of the maximum clot polymerization to the threshold of 50% of fibrinolysis and determines the overall fibrinolytic properties of the clot. All turbidimetry data were analysed with the Shiny App tool [21].

Statistical analyses

Baseline variables were summarized as mean (SD) and proportions. Hemostatic biomarkers were evaluated as arithmetic means (SD). In the primary analysis, we tested for differences in measurements before and after supplementation with vitamin D using paired T-tests, therefore estimating the mean absolute change between those 2 times. Relative differences in geometric means and analyses with categorical variables were also reported. In secondary analyses,

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the absolute change in 25-OHD measurements after supplementation was correlated to the absolute change in hemostatic measurements, using linear regression. Finally, we also explored the following subgroups: according to baseline 25-OHD levels (in tertiles), baseline PTH levels (secondary hyperparathyroidism vs. not), age (<43 vs. ≥43 years), sex and co-morbidities (hypertension, diabetes and/or cardiovascular disease).

There was a minimal amount of missing values, and no imputation was performed. Outliers were assessed graphically and excluded in sensitivity analyses to assess their impact on the primary findings. We also repeated the primary analyses with a permutation test for comparison of means (no assumption of the distribution of data) instead of T-tests, without any difference in statistical differences.

Statistical significance was set at an alpha level of 0.05. All analyses were conducted on Stata 11 (StataCorp, College Station, USA).

Results

From 6.2016 to 6.2017, we screened 2015 measurements of 25-OHD, of which 363 were <25nmol/L (prevalence of 18%). After exclusion, mostly because of ongoing supplementation of vitamin D or obesity, 55 participants were included, of whom 48 completed the study (Figure 1).

The 48 participants had a mean age of 44 years (range 23-74 years) and were of diverse race/ethnicity (Table 1). 25/48 had at least one cardiovascular risk factor. One participant had a history of CVD, and none of VTE.

Vitamin D supplementation significantly and greatly increased mean levels of 25-OHD, from 17.9nmol/L±4.6 to 62.5nmol/L±20.7 (P<0.0001), after an average of 6.4±3.0 weeks of study time (Table 2). The increase in 25-OHD levels ranged from

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7nmol/L to 99nmol/L. While all participants had severe baseline deficiency (<25mol/L), at follow-up 12 had normal levels (≥75nmol/L), 23 had insufficient levels (50-75nmol/L), 12 had deficient levels (25-50nmol/L) and 1 had persistent severely deficient levels. PTH levels decreased accordingly. 26/48 and 12/48 had secondary hyperparathyroidism, defined as PTH >6.8pmol/L, at baseline and at follow-up, respectively. Serum calcium remained unchanged at normal values.

We observed differences in thrombin generation measurements, after supplementation with cholecalciferol (Table 2). Mean endogenous thrombin potential (ETP) was decreased by 95.4nM*min (95%CI -128.9 to -62.8, p<0.001), corresponding to a relative reduction of geometric means of 6.8% (95%CI 4.6-9.0). Peak values were smaller (-15.1, 95%CI -23.3 to -6.8, p<0.001) and both the lag time and the time to peak increased after supplementation. In turbidimetry assays, the maximum clot density was lower after supplementation, with a relative reduction of geometric means of 8.5% (95%CI -13.1 to -3.6). One participant had a large reduction of ETP (25.2%) after supplementation. Excluding this possible outlier did not affect the results or their statistical significance.

Measurements of fibrinolysis were unchanged after supplementation, including both clot lysis time and the AUC of fibrinolysis (data not shown). Coagulation times (PT and aPTT) and levels of fibrinogen, PAI-1 and homocysteine levels remained stable during the study (Table 2).

Neither the absolute nor the relative change of ETP were related to the absolute continuous change in 25-OHD levels, in univariate analysis (p=0.43 and p=0.48, respectively). In subgroup analyses, we explored the absolute change of ETP across tertiles of magnitude of change of 25-OHD levels (Figure 2A) and between participants with normal and elevated baseline PTH (secondary

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hyperparathyroidism, Figure 2B), without suggestion of effect modification. Similarly, there was no modification of the positive findings for ETP in subgroups of age, sex and co-morbidities (data not shown).

During the study time, no participants suffered from a cardiovascular or VTE event or changed their co-medication apart from vitamins in a few.

Discussion

In this prospective cohort of outpatients with severe vitamin D deficiency, the supplementation with high-dose calcidiol was associated with reduced *in vitro* thrombin generation and decreased clot density, without change in fibrinolytic times. These findings may suggest that vitamin D supplementation decrease a prothrombotic profile induced by severe vitamin D deficiency.

Three other studies evaluating changes in thrombin generation after calcidiol have generated conflicting findings, including two negative studies. In a Norwegian clinical trial, 158 obese participants were randomized to 40,000IU of weekly cholecalciferol or placebo supplementation for 12 months. In the vitamin D group, mean 25-OHD levels increased from 61.8 to 145.5nmol/L.[11] The observed lack of change in thrombin generation in this study may come from the selection of obese patients without a vitamin D deficiency at baseline. The second negative study was a recent Dutch prospective cohort, in whom 59 patients with severe 25-OHD were treated with a 2-month oral cholecalciferol supplementation of 900,000IU. While the 25-OHD levels increased dramatically from 20nmol/L to an average of 108nmol/L, thrombin generation parameters and clot lysis time remained similar.[15] Our discrepant positive results may arise from a stricter selection of participants without active cardiovascular disease (albeit with cardiovascular risk factors) and

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antithrombotic drugs, and a different condition of the thrombin generation assay (using 1pM of tissue factor). A third study treated 53 Israeli healthy volunteers with 25-OHD <50nmol/L with 2000IU of daily cholecalciferol supplementation for 90 days, increasing their mean 25-OHD levels from 32 to 52nmol/L. Contrary to our findings, thrombin generation was greater after supplementation, with greater ETP and peak levels.[14] We hypothesize, given this body of evidence, that a reversible prothrombotic hemostatic environment may only be observed in patients with severe vitamin D deficiency (25-OHD levels <25nmol/L), without primary hyperparathyroidism or CVD, who were specifically included in our cohort. This hypothesis may be supported by the secondary analyses of the Women's Health Initiative, in which supplementation with 400IU of daily vitamin D and calcium reduced the risk of VTE in the small subgroup of women with 25-OHD <25nmol/L, although this may have been a chance finding.[3]

In the general population, two recently published large trials have demonstrated the lack of influence of cholecalciferol supplementation on arterial cardiovascular events. The VITAL study randomized 25,871 American >50-55 years old participants to 2000IU of cholecalciferol or placebo.[6] A New Zealander study randomized 5110 >50 years old participants to 200,000IU then 100,000IU monthly of cholecalciferol or placebo.[7] No changes in the risk of major cardiovascular outcomes were noted after median follow-ups of 5.3 and 3.3 years, respectively. In both studies, vitamin D deficiency (<50nmol/L) was under-represented (12.7% and 30%), with mean baseline 25-OHD levels of 77nmol/L and 64nmol/L, respectively. Therefore, for severe vitamin D deficiency (<25nmol/L), no firm conclusions can be drawn at this point.

The influence of secondary hemostasis (i.e. coagulation) on the risk of atherosclerotic disease is well documented. Anticoagulation with vitamin K antagonists is an effective secondary prevention of coronary heart disease events, albeit at a cost of bleeding. Recently, the COMPASS trial established the role of low-dose anticoagulants (rivaroxaban), in addition to aspirin, on the risk of cardiovascular death, stroke and myocardial infarction, among participants with stable CVD.[22] On top of the clear link to venous thromboembolism, modifications of the hemostatic environment may therefore represent a plausible mechanism explaining an elevated cardiovascular arterial risk associated with severe vitamin D deficiency.

We studied biological endpoints and no conclusive clinical implications should be drawn. Both high thrombin generation and thrombin peak levels are associated with the risk of a first VTE, suggesting that thrombin generation may represent a valid intermediate phenotype.[23, 24] However, whether the 95nM*min or the 6.8% decrease in ETP with supplementation translates into fewer VTE events is speculative. As a comparison, the G20210 prothrombin polymorphism causes a 3-fold increased risk of incident VTE and is associated with a 422nM*min or 20% difference in ETP, using the same thrombin generation assay.[25]

Abnormal fibrin clot structure, in particular increased fibrin fiber density and resistance to fibrinolysis are reported to be linked to CVD.[16] However, several modulators such as blood cells interactions, blood flow conditions and endothelium can modify the fibrin clot network *in vivo*.[26] It is likely that these effect (e.g. effects of vitamin D on endothelium) are not properly detected by *ex vivo* studies.[27] Similarly, the *in vitro* capacity of plasma to generate thrombin over time (ETP) reflect of potential thrombin generation that must be distinguished from in vivo markers of

thrombin generation such as prothrombin fragments 1 and 2, thrombin anti-thrombin complexes and fibrinopeptide A.

The strengths of our study include the assessment of global hemostasis assays and fibrin clot structure and the restriction to patients suffering from severe vitamin D deficiency, which brings supplementary data to this field. The main limitation of our findings is the lack of control group, which precludes us from drawing any definite causal conclusions. However, during the short follow-up of our study (6.8 weeks), there was almost no new drug prescribed (only vitamins) and no change in contraceptives.

In conclusion, our findings support the concept of a relationship between severe vitamin D deficiency and a prothrombotic phenotype. Given the recent negative evidence from large trials of vitamin D supplementation in the general population, the influence of vitamin D status on hemostasis may be restricted to individuals with a severe vitamin D deficiency.

Declaration of interest, Funding and Acknowledgments

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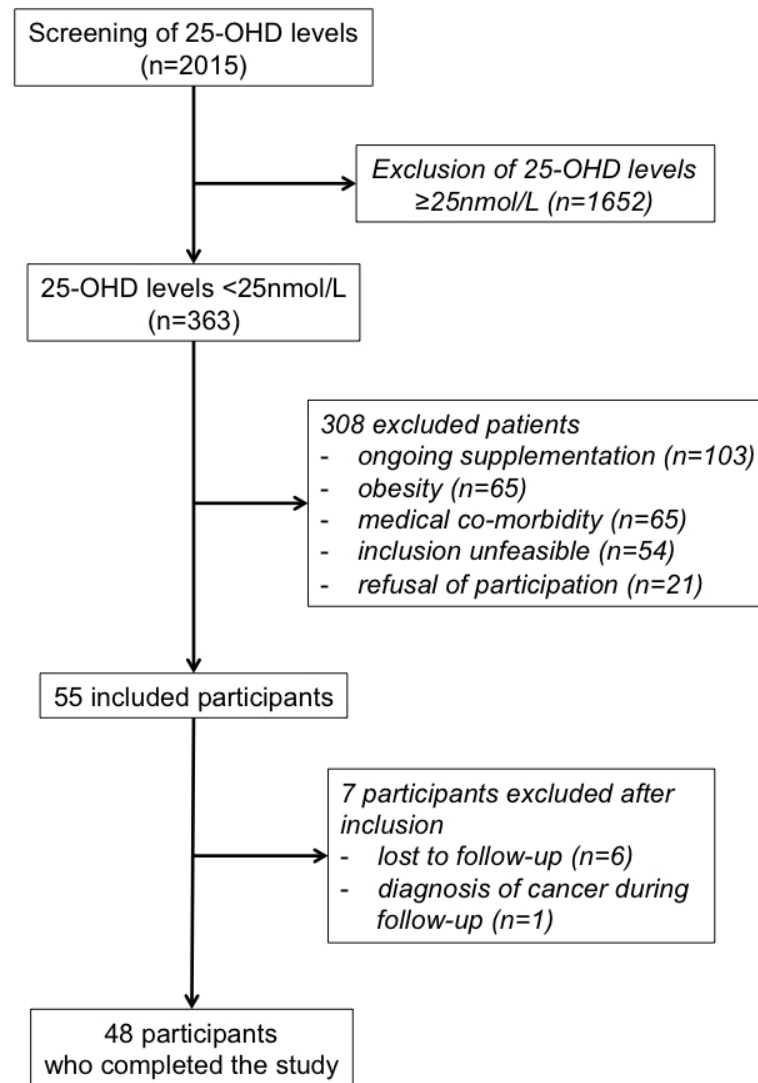
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Figure 1. Flow-chart of the study

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Figure 2. Boxplots (median and 25/75th percentile) of endogenous thrombin potential.

2A. In subgroups of tertiles of change of 25-OHD levels over the study period (1=7-34mM ; 2=35-52mM ; 3=53-99mM)
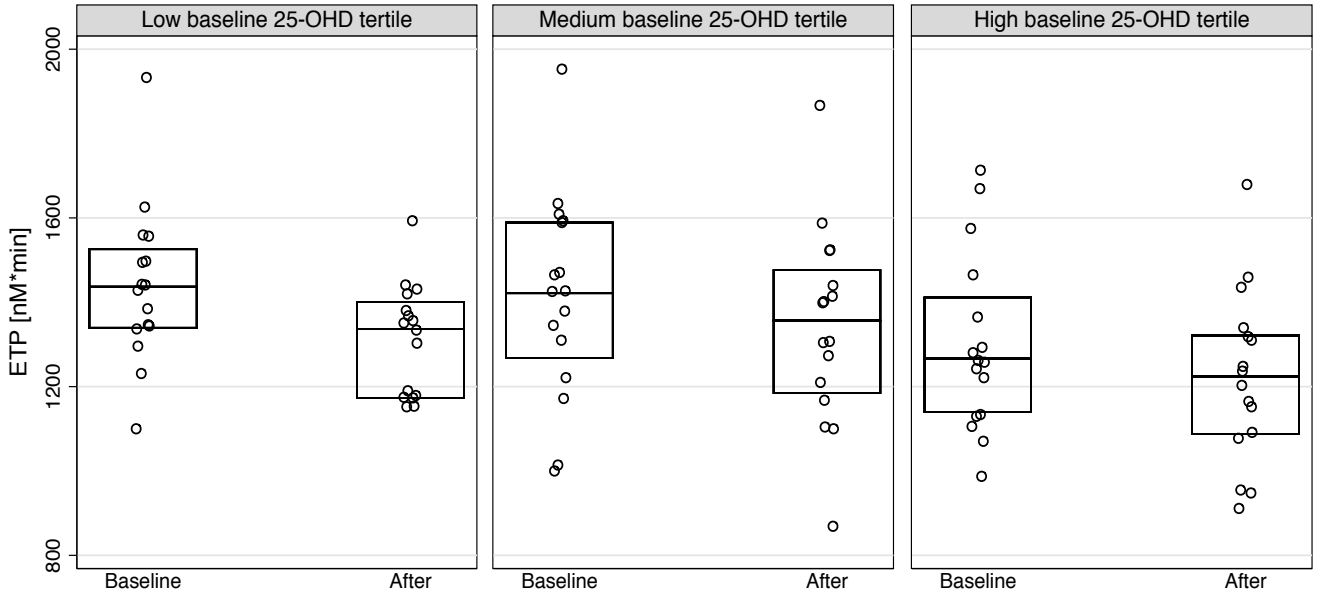
2B. In subgroups of baseline PTH

Legend: Results are statistically different (P<0.05) in all 5 subgroups.

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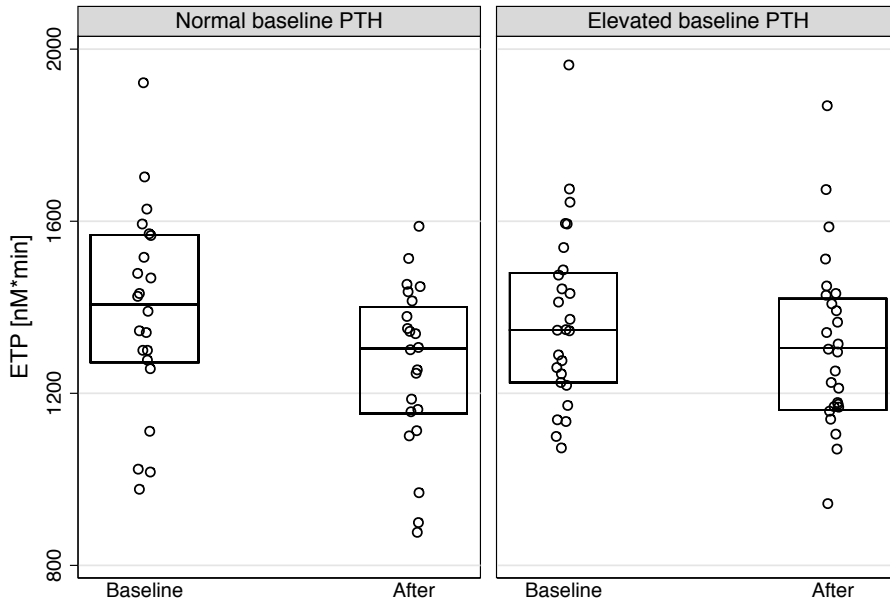
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Flow-chart of the study

254x338mm (72 x 72 DPI)

2a

2b

Table 1. Baseline characteristics of the participants (n=48)

Baseline characteristics	n or mean (SD) (N=48)
Men	21
Age, years	43.8 (13.8)
Race	
White	17
Hispanic White	19.
Black	7.
Asian	5
BMI, kg/m^2	24.2 (3.4)
Current smoker	14
Diabetes	6
Hypertension	11
Mean SBP/DBP, mmHg	124/76 (16/10)
Cardiovascular disease	1
Prior fracture	9 *
Current use of	
Anti-hypertensive	12
Statins	7
Folates	7
Estrogenic contraceptive	0
Progestogen-only contraceptive	4
CKD-EPI GFR, ml/min	107 (17)
Hemoglobin, g/L	138.7 (16.5) **
Platelets, G/L	287.8 (66.1) **
Prothrombin time, %***	92.3 (8.6)
aPTT***	30.0 (3.2)
Fibrinogen, g/L***	3.1 (0.7)

*1 and **7 missing values; ***Normal ranges: Prothrombin time >70%, aPTT 26-37 sec, fibrinogen 1.5-3.5 g/L.

Table 2. Mean levels of mineral metabolism and hemostatic biomarkers before and after supplementation.

	Baseline (SD)	After supplementation (SD)	Absolute difference (95%CI)	P value
25D, nmol/L	17.9 (4.6)	62.5 (20.7)	+44.5 (+38.8 to +50.3)	<0.001
PTH, pmol/L	7.6 (2.9)	6.2 (2.5)	-1.4 (-0.7 to -2.1)	<0.001
Calcium, mmol/L	2.29 (0.08)	2.29 (0.08)	0.00 (-0.02 to 0.02)	0.72
Thrombin Generation				
ETP, nM*min	1384.3 (218.4)	1288.9 (197.2)	-95.4 (-127.9 to -62.8)	<0.001
Peak, nM	221.5 (46.5)	206.5 (45.7)	-15.1 (-23.3 to -6.8)	<0.001
Lag time, min	2.99 (0.50)	3.24 (0.53)	+0.25 (+0.12 to +0.37)	<0.001
Time to peak, min	6.41 (0.98)	6.63 (0.92)	+0.22 (+0.03 to +0.41)	0.027
Fibrinolysis				
Clot lysis time, min *	16.6 (5.5)	17.0 (6.6)	+0.4 (-1.3 to +2.1)	0.62
Fibrin polymerization				
Maximum	0.43 (0.08)	0.40 (0.08)	-0.04 (-0.06 to -0.02)	0.001
Prothrombin time, %	92.3 (8.6)	91.9% (8.6)	-0.4 (-2.2 to 1.4)	0.65
aPTT, sec	30.0 (3.2)	30.0 (3.2)	0.0 (-0.5 to 0.5)	0.95
Fibrinogen activity, g/L	3.1 (0.7)	3.0 (0.7)	0.0 (-0.2 to 0.1)	0.64
PAI-1	12.9 (8.0)	14.0 (13.0)	+1.1 (-1.7 to + 3.9)	0.42
Homocysteine **	14.7 (14.8)	12.0 (4.9)	-2.7 (-6.4 to +1.1)	0.15

*n=47
**n=45

ETP = endogenous thrombin potential ; OD = optical density ; aPTT = activate partial thromboplastin time ; PAI-1 = plasminogen activator inhibitor-1