**Percorso d'eccellenza**
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# Modulation of 1,25(OH)2D levels in cardiovascular system.

*Genetic regulation, impact on coronary artery disease, inflammation, platelet function and periprocedural myocardial injury after percutaneous coronary interventions*

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*“Learning never exhausts the mind”*

*Leonardo da Vinci*

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# Part 1

## Introduction and rationale

Vitamin D deficiency has achieved, in the last years, a dramatic prevalence in Western counties, ranging over 50% of prevalence in the population, (1), as a consequence of pollution, the progressive ageing and the increased frequency of chronic disorders as renal failure.

Vitamin D is a fat-soluble vitamin, representing not only the principal modulator of calcium and bone homeostasis, but also a key-hormone, regulating the transcription of about 2,000 to 8,000 genes (2).

Recent attention has been addressed to the cardiovascular effects of vitamin D, (3), modulating function, proliferation and differentiation of cardiomyocytes, endothelial cells, vascular musculature and the immunity processes involved in the regulation of cardiovascular health. In fact, hypovitaminosis D has been involved in the pathogenesis of hypertension, diabetes mellitus, metabolic disorders, but it has also been associated to ventricular hypertrophic remodelling, vascular wall degeneration and its athero- thrombotic complications (4,5).

Different studies and a recent meta-analysis have demonstrated the clear negative prognostic impact of vitamin D deficiency on all-cause and cardiovascular mortality, (6), showing an inverse linear relationship with an increase in cardiovascular risk for every 10 ng/ml reduction in 25(OH)D. However, clinical trials providing vitamin D supplementation have failed to demonstrate any significant benefit in cardiovascular outcomes (7). Several explanations have been provided for such findings, including inadequate study design and factors modulating vitamin D repletion or differential activation. In fact, the majority of the studies so far conducted were not powered for the evaluation of cardiovascular endpoints, and moreover the therapeutic strategy was insufficient

for the restoration of adequate levels of vitamin D, therefore rendering inconclusive the majority of the data.

Moreover, particular attention has been focused on the role of genetics, that could modulate the transportation and the systemic levels of vitamin D, but also its transformation to the hydroxylated form, with hormonal effect. In fact, two common genetic variants of the Vitamin D Binding Protein (VDBP), vitamin D transporter, have been held responsible for more than 10% of the interindividual variability in the circulating levels of 25(OH)D, conditioning its bioavailability and peripheral effects (8). In fact, the variations in the DBP originally referred to as GC1F, GC1S, and GC2 were first reported more than 50 years ago and can condition its affinity for 25(OH)D and, therefore, the quote of "free" functional vitamin.

In addition, five single nucleotide polymorphisms have been described for vitamin D receptor (VDR), partially combined among them in consequence of linkage disequilibrium, to generate specific haplotypes with consequences on the receptor's signaling and effects (9).

Among these variants, *Cdx* and *GATA* are located at the promoter of the gene, controlling the transcription of the receptor and then the effectiveness of calcitriol signaling, *FokI* is responsible of a change in the coding region, conditioning the binding of vitamin D to VDR, whereas the variants *BsmI, ApaI* e *TaqI* fall in the 3'UTR of the gene, potentially conditioning the stability of the mRNA and then the achievement of the transcription product (10). *BsmI,* in particolar, has been positively linked to cardiovascular risk, causing a loss of the cardioprotective effects of vitamin D and a disregulation in the transformation of vitamin D into its active form. In fact, the complex vitamin D-VDR controls the inhibition of CYP27B1 (the 1-α hydroxylase activating vitamin D) and activates CYP24A1 (the 24-α hydroxylase responsible for the degradation of activated calcitriol).

Moreover, genetic variants of these hydroxylation enzymes have been described, potentially modulating the levels of the activated 1,25(OH)2D and therefore the final action of the hormone, although their impact on vitamin D signaling is still matter of debate (11).

A more intriguing hypothesis, however, recently proposes that many effects of 25(OH)D could be the consequence of a local, autocrine or paracrine activation of vitamin D (12). Such kind of production derives from the differential expression of CYP27B1 in different cells types, as endothelial cells, myocytes, cardiomyocytes and macrophages, that can generate a transient and local increase in the intravascular concentrations of activated vitamin D (13).

In effect, Dickie *et al.* have demonstrated that the release of pro-inflammatory cytokines can induce an autocrine/paracrine production of 1,25(OH)2D in macrophages and lymphocytes attracted to the inflammation site, resulting in a reduction of inflammation and a preferential production of anti-infiammatory factors as IL4 or IL10, that stimulate the $T_{reg}$ e $T_{h2}$ lymphocytes (14). This mechanism has been proposed to explain how 1,25(OH)2D can prevent the production of foam cells in diabetic patients, contrasting the generation of the atheromasic plaque (15).

Nevertheless, the cardiovascular impact of the differential homeostasis of vitamin D is poorly understood. Anyhow, a relevant role could be hypothesized especially in the context of acute coronary syndromes or among patients undergoing percutaneous coronary interventions. In fact the pro-thrombotic, pro-inflammatory and pro-oxidant setting might enhance the importance of the activity of vitamin D, both at a local level and for its systemic implications, modulating platelet reactivity and circulating cytokines. In addition, no study has so far addressed of 1,25(OH)2D in coronary artery disease (CAD).

In the present thesis we will confirm and re-assess the mechanisms involving hypovitaminosis D in the pathogenesis of atherosclerosis and in the regulation of platelet hyper-reactivity and thrombotic risk, with a focus on those genetic factors that could condition the interindividual

difference in its bioavailability and effectiveness. In addition, we will assess the relationship between the levels of 25(OH)D and 1,25(OH)2D, attempting to identify those clinical and genetic the determinants of the activation of vitamin D, trying to provide new targets for the therapeutic strategies of supplementation and shed light on the reasons for negative findings of previous trials with vitamin D.

## References


1. Lavie CJ, DiNicolantonio JJ, Milani RV, O'Keefe JH. Vitamin D and Cardiovascular Health. Circulation. 2013;128:2404-2406.
2. Gil Á, Plaza-Diaz J, Mesa MD. Vitamin D: Classic and Novel Actions. Ann Nutr Metab. 2018;72(2):87-95
3. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, Galasso G, De Luca G. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014;44(7):634-42
4. Kunadian V, Ford GA, Bawamia B, Qiu W, Manson JE. Vitamin D deficiency and coronary artery disease: A review of the evidence. Am Heart J 2014;167:283-91
5. Stach K, Kalsch AI, Nguyen XD, et al. 1alpha,25-dihydroxyvitamin d3 attenuates platelet activation and the expression of vcam-1 and mt1-mmp in human endothelial cells. Cardiology 2011;118:107-15
6. Wang C. Role of vitamin d in cardiometabolic diseases. J Diabetes Res. 2013;2013:243934
7. Mao Mao PJ, Zhang C, Tang L, Xian YQ, Li YS, Wang WD, Zhu XH, Qiu HL, He J, Zhou YH. Effect of calcium or vitamin D supplementation on vascular outcomes: a meta-analysis of randomized controlled trials. Int J Cardiol. 2013 ;169(2):106-11
8. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, Powe NR, Thadhani R. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013;369(21):1991-2000
9. Fang Y, van Meurs JB, d'Alesio A, Jhamai M, Zhao H, Rivadeneira F, Hofman A, van Leeuwen JP, Jehan F, Pols HA & Uitterlinden AG. Promoter and 30-untranslated-region haplotypes in the vitamin D receptor gene predispose to osteoporotic fracture: the Rotterdam study. American Journal of Human Genetics 2005 ; 77 :807–823
10. De Jongh RT, Lips P, Rijs JK, van Schoor NM, Kramer NH, Vandenbroucke JP, Dekker OM. Associations between vitamin D receptor genotypes and mortality in a cohort of older Dutch individuals. European Journal of Endocrinology, 164: 75–82
11. Levin GP, Robinson-Cohen C, de Boer IH, Houston DK, Lohman K, Liu Y, Kritchevsky SB et al. Genetic Variants and Associations of 25-Hydroxyvitamin D Concentrations With Major Clinical Outcomes
12. Richart T, Li Y, Staessen JA. Renal versus extrarenal activation of vitamin D in relation to atherosclerosis, arterial stiffening, and hypertension. Am J Hypertens. 2007;20:1007–1015
13. Norman PE, Powel JT. Vitamin D and Cardiovascular Disease. Circ Res. 2014;114:379-393
14. Dickie LJ, Church LD, Coulthard LR, Mathews RJ, Emery P, McDermott MF. Vitamin D3 down-regulates intracellular Toll-like receptor 9 expression and Toll-like receptor 9-induced IL-6 production in human monocytes. Rheumatology (Oxford). 2010;49:1466–1471.
15. Riek AE, Oh J, Sprague JE, Timpson A, de las Fuentes L, Bernal- Mizrachi L, Schechtman KB, Bernal-Mizrachi C. Vitamin D suppression of endoplasmic reticulum stress promotes an antiatherogenic monocyte/macrophage phenotype in type 2 diabetic patients. J Biol Chem. 2012;287:38482–38494

Part 2

# *Vitamin D in coronary artery disease*

# Chapter 2:

## Vitamin D deficiency is independently associated with the extent of coronary artery disease

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### Abstract

**Background.** Vitamin D (25-OH D3) deficiency represents a rising social and economic problem in Western countries. Vitamin D has been recently reported to modulate inflammatory processes, endothelium and smooth muscle cell proliferation and even platelet function, thus potentially modulating atherothrombosis. Great interest has been addressed on its impact on cardiovascular outcome, with contrasting results. The aim of current study was to evaluate the relationship between 25-OH D3 and the extent of coronary artery disease in a consecutive cohort of patients undergoing coronary angiography.

**Methods.** Patients undergoing elective coronary angiography were included in a cross-sectional study. Fasting samples were collected for 25-OH D3 levels assessment. Significant CAD was defined as at least 1 vessel stenosis > 50%, while severe CAD as left main and/or trivessel disease, as evaluated by Quantitative Coronary Angiography.

**Results**. Hypovitaminosis D was observed in 70.4 % out of 1484 patients. Patients were divided according to vitamin D tertiles (<9.6; 9.6-18.4; ≥18.4). Lower vitamin D levels were associated with age, female gender (p<0.001, respectively), renal failure (p=0.05), active smoking (p=0.001), acute coronary syndrome at presentation (p<0.001), therapy with calcium-antagonists (p=0.02) and diuretics (p<0.001), less beta-blockers (p=0.02) and statins (p=0.001) use. Vitamin D directly related to haemoglobin (p<0.001) and inversely with platelet count (p=0.002), total and LDL cholesterol (p=0.002 and p<0.001) and triglycerides (p=0.01). Vitamin D did not influence angiographic features of coronary lesions, but was associated with higher prevalence of left main or right coronary artery

disease (p=0.03). Vitamin D deficiency was significantly associated with higher prevalence of CAD (adjusted OR[95%CI]=1.32[1.1-1.6],p=0.004) and severe CAD (adjusted OR[95%CI]= 1.18[1-1.39], p=0.05).

Conclusion. In patients undergoing coronary angiography hypovitaminosis D was observed in the vast majority of patients. Vitamin D deficiency is significantly associated with the prevalence and extent of CAD, especially for patients with values < 10 ng/ml. Therefore, future large studies are needed to evaluate whether vitamin D supplementation may prevent CAD and its progression.

Keywords: vitamin D, coronary artery disease; coronary angiography

## Introduction

Cardiovascular disease represents the leading cause of mortality in developed countries. Great reduction in mortality has been achieved by improvement in myocardial revascularization techniques (1,2), however, the results are still unsatisfactory in high-risk subgroups. Therefore, large interests have been focused on the identification of new risk factors for Coronary Artery Disease (CAD) (3) and its prevention.

Calcium metabolism disorders, and in particular vitamin D (25-hydroxy -cholecalciferol, 25-OHD3) deficiency, represent a rising problem, whose social and economic impact is growing due to aging of the population. Vitamin D has received, in the last few years, great interests for its multiple effects on inflammatory system and potential role in atherothrombosis. Hypovitaminosis D has been related to endothelial dysfunction and enhanced risk of cardiovascular disease (4-6). In fact, Vitamin D receptor has been identified on the surface of smooth muscle cells (7), endothelial cells and cardiomyocytes, but also on inflammatory cells, controlling their proliferation and differentiation (8), and even in platelets, thus potentially influencing thrombosis (9). Furthermore, independent association has been demonstrated between vitamin D deficiency and cardiovascular risk factors, such as hypertension (10), diabetes mellitus (11), obesity (12), metabolic syndrome(13). These associations may contribute to explain the observed relationship between vitamin D and atherosclerosis. The aim of the present study is to evaluate the relationship between 25-OH D3 and the extent of CAD in a consecutive cohort of patients undergoing coronary angiography.

## Methods

Consecutive patients undergoing coronary angiography between September 2009 and June 2013 at the Ospedale “Maggiore della Carità”, Novara, Italy were eligible to participate in a cross-sectional study. Informed consent before angiography was the only required inclusion criterium. Patients receiving vitamin D supplementation at admission or those whose vitamin D status was unavailable

were excluded. The study was approved by our local Ethical Committee. All demographic and clinical data were prospectively collected in a dedicated database. Hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg or if the individual was taking antihypertensive medications (14). Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycaemia > 126 mg/dL or HbA1c > 6.5%. Chronic renal failure was considered for history of renal failure or an admission glomerular filtration rate (GFR) < 60 mol/min/1.73m2 by MDRD (Modification of Diet in renal Disease) formula.

### Biochemical measurements

Blood samples were drawn at admission in patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography. Glucose, creatinine, glycosylated haemoglobin and lipid profile were determined by standard methods. Vitamin D dosing was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for 25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference (15). Severe hypovitaminosis D was considered for values < 10 ng/ml as previously reported (16).

### Coronary angiography

Coronary angiography (carried our by Siemens AXIOM ARTIS *d*TC, Erlangen, Germany) was routinely performed by the Judkins technique using 6-French right and left heart catheters. Quantitative coronary angiography was performed by two experienced interventional cardiologists who had no knowledge of the patients' vitamin D status, by an automatic edge-detection system for Quantitative Coronary Angiography (Siemens Acom Quantcor QCA, Erlangen, Germany) (17)). After a visual inspection of the coronary artery, the frame of optimal clarity was selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, analysed arterial segment with coronary lesion was defined by moving the cursor from the proximal to the distal part of coronary artery to ensure adequate determination of reference diameter. Minimal

luminal diameter, reference diameter, percent diameter stenosis, and length of the lesion were measured. Significant CAD was defined as the presence of at least 1 coronary stenosis more than 50%. Severe CAD was defined as the presence of three-vessel disease and/or left main disease. For patients who had previously undergone percutaneous coronary interventions, the treated lesion was considered as significantly diseased vessel. In previously bypassed patients, both native arteries and grafts were taken into account in the evaluation of extension of coronary artery disease (number of diseased vessels).

### Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Patients were grouped according to tertiles of Vitamin D. A trend analysis was performed as previously described (18). In particular, we used polynomial contrasts in case of a continuous variable, whereas we assessed the linearity assumption by including new variables (the upper three quartiles vs. the lowest quartile of vitamin D) in the regression model and plotting the estimated logistic regression coefficients versus the quartile midpoints of vitamin D in case of a categorical variable. Multiple logistic regression analysis was performed to evaluate the relationship between 25OH-D3 and CAD, after correction for baseline confounding factors (all baseline characteristics that significantly (p value $\leq$ 0.05) differed between groups), that were entered in the model in block. Log-transformation was applied to non-normally distributed variables (as evaluated by the Kolmogorov-Smirnov test) before entering into the logistic regression model. A p value $\leq$ 0.05 was considered statistically significant.

**Table 1.** Clinical characteristics according to Vitamin D tertiles

| **Baseline clinical characteristics** | I tertile<br>< 9.6<br>N= 482 | II tertile<br>9.6-18.4<br>N= 495 | III tertile<br>≥ 18.4<br>N= 507 | P value |
|---|---|---|---|---|
| Age (mean±SD) | 68.9±12 | 66.8±11.4 | 67.4±11.3 | <0.001 |
| Male Sex (%) | 62 | 74.7 | 74.4 | <0.001 |
| Hypercholesterolemia(%) | 59.5 | 57.7 | 54.9 | 0.33 |
| Diabetes mellitus (%) | 36.9 | 32.3 | 33.3 | 0.29 |
| Renal failure (%) | 18.9 | 10.1 | 14.4 | 0.05 |
| Family history of CAD (%) | 25.9 | 30.4 | 32.1 | 0.13 |
| Smokers (%) | | | | 0.001 |
| Active smokers (%) | 24.8 | 23.2 | 16.6 | |
| Previous smokers (%) | 19.9 | 24.6 | 28.8 | |
| Hypertension (%) | 75.6 | 71.9 | 72.9 | 0.39 |
| History of MI (%) | 23.4 | 23.4 | 20.3 | 0.25 |
| Previous PCI (%) | 23.6 | 28.4 | 28.2 | 0.17 |
| Previous CABG (%) | 13.4 | 9.1 | 11.1 | 0.26 |
| Previous CVA (%) | 7.4 | 4.9 | 5.3 | 0.18 |
| **Indication to angiography** | | | | <0.001 |
| Stable angina/ silent ischemia (%) | 14.8 | 23.2 | 27.3 | |
| STEMI/ACS (%) | 61.5 | 60.2 | 56.9 | |
| Other (%) | 23.7 | 16.6 | 15.7 | |
| ACE inhibitors(%) | 38.1 | 38.3 | 33.7 | 0.15 |
| ARB (%) | 22.5 | 19.2 | 21.7 | 0.75 |
| Beta blockers (%) | 47 | 52.5 | 54.3 | 0.02 |
| Nitrates (%) | 31.1 | 31.7 | 32.1 | 0.74 |
| Statins (%) | 41.5 | 51.8 | 52.7 | 0.001 |
| Calcium antagonists (%) | 23 | 17.8 | 17.2 | 0.02 |
| Diuretics (%) | 37.6 | 27.7 | 23.2 | <0.001 |
| ASA (%) | 54.4 | 58.5 | 59.4 | 0.11 |
| Clopidogrel (%) | 17.2 | 19.4 | 20.5 | 0.65 |
| **Biochemistry parameters** | | | | |
| Platelets (10^6/ml; mean± SD) | 226.8±81.4 | 218±59.8 | 211.7±61.3 | 0.002 |
| Haemoglobin ( g/dl) | 13.1±1.8 | 13.5±1.6 | 13.6±1.6 | <0.001 |
| WBC (10^3/ml;mean± SD) | 8±2.7 | 7.9±2.4 | 7.6±2.7 | 0.08 |
| HDL cholesterol (mg/dl) | 42.7±13.4 | 43.3±12.8 | 43.9±13.6 | 0.39 |
| Total cholesterol (mg/dL) | 169.7±45.1 | 163.1±42.8 | 160.3±37.6 | 0.002 |
| LDL cholesterol (mg/dL) | 101.5±46.1 | 93.7±38.6 | 91.8±33.7 | <0.001 |
| Triglycerides (mg/dl) | 141.7±95.5 | 136.9±78.6 | 126.6±70.4 | 0.01 |
| Glycaemia (mg/dL) | 127.2±55.9 | 123.4±50.2 | 123.4±40.2 | 0.27 |
| Creatinine (mg/dL) | 1.1±0.82 | 1.05±0.68 | 1.1±0.72 | 0.48 |

CAD = Coronary Artery Disease; MI = Myocardial Infarction; CVA = Cerebrovascular Accident; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; LDL = Low-Density Lipoproteins.

## Results

Between September 2009 and June 2013, 1509 patients underwent coronary angiography at our institution. Among them, 25 patients were taking chronic vitamin D supplementation and were therefore excluded. Thus, 1484 patients were enrolled.

Hypovitaminosis D (< 20 ng/mL) was observed in 1045 patients, (70.4% of the total population). Vitamin D levels underwent seasonal variation, with significant differences ($p<0.001$) between spring/summer months (April to September, 18.05 ± 11ng/mL) as compared with autumn/winter (October to March, 13.6 ± 8.3 ng/mL) months.

Patients were divided in three groups according to tertiles of Vitamin D (< 9.6, 9.6-18.4, > 18.4 ng/mL, respectively). Baseline demographic and clinical characteristics of the three groups are shown in Table 1. Vitamin D was associated to age ($p<0.001$), female gender ($p<0.001$), renal failure ($p=0.05$), active smoking ($p=0.001$), presentation with acute coronary syndrome ($p<0.001$), therapy at admission with calcium-antagonists ($p=0.02$) and diuretics ($p<0.001$), as well as with less frequent use of beta-blockers ($p=0.02$) and statins ($p=0.001$). Vitamin D directly related with haemoglobin levels ($p<0.001$) and inversely with platelet count ($p=0.002$), total and LDL cholesterol ($p=0.002$ and $p<0.001$, respectively) and triglycerides ($p=0.01$).

Table 2 displays main angiographic features, that did not differ according to vitamin D levels, with the exception of higher prevalence of left main ($p=0.03$) or right coronary artery disease ($p=0.03$).

No significant difference was found between vitamin D deficiency tertiles and general coronary artery disease prevalence (79.1% vs 75.8 % vs 75.4%; $p=0.17$, OR [95%CI]= 1.11[0.96-1.28], $p = 0.18$), as depicted in Figure 1A. However, as shown in Figure 1B, lower vitamin D deficiency tertiles were significantly associated with a higher prevalence of severe coronary artery disease (34.4% vs 27.3% vs 27.0%, $p = 0.01$, OR[95%CI] = 1.19 [1.04-1.37], $p = 0.01$).

Table 2. Angiographic characteristics according to Vitamin D tertiles (per lesion)

| Angiographic features | I tertile < 9.6 N= 904 | II tertile 9.6-18.4 N= 845 | III tertile ≥ 18.4 N= 819 | P value |
|---|---|---|---|---|
| Left main disease (%)§ | 13.2 | 9.3 | 9 | 0.03 |
| LAD (%)§ | 54.3 | 55.1 | 54.1 | 0.94 |
| CX (%)§ | 39.8 | 41.5 | 41.7 | 0.56 |
| RCA (%)§ | 50.4 | 46.7 | 43.9 | 0.03 |
| Type C Lesion (%) | 32 | 32.8 | 33.8 | 0.41 |
| Lesion length (mm) | 20.9±13.9 | 22±13.4 | 21.7±12.7 | 0.27 |
| Percent stenosis (%) | 86±14.9 | 86.8±13.2 | 87.2±13.8 | 0.54 |
| Reference Diameter (mm) | 2.9±0.89 | 3±2.2 | 2.9±1.2 | 0.26 |
| Proximal vessel tortuosity (%) | 1.6 | 1.8 | 2.1 | 0.43 |
| Calcifications (%) | 20.3 | 21.6 | 18.5 | 0.38 |
| Chronic occlusion (%) | 19 | 15.1 | 16.6 | 0.18 |
| Restenosis (%) | 3 | 5.8 | 4.3 | 0.17 |
| Thrombus (%) | 3.4 | 4.7 | 3.3 | 0.64 |
| TIMI Flow | | | | 0.83 |
| 3 | 68.9 | 71.5 | 70.7 | |
| 2 | 8 | 7.1 | 5 | |
| 1 | 4.2 | 3.3 | 3.3 | |
| 0 | 18.9 | 18.1 | 21 | |

§ Per patient definition

*LAD= Left Anterior Descending coronary artery; Cx= Circumflex coronary artery; RCA= Right Coronary Artery; TIMI= Thrombolysis in Myocardial Infarction;*

Figure 1. Bar graphs showing the relationship between vitamin D tertiles values and the prevalence of coronary artery disease (CAD) Figure 1A (upper graph), and between vitamin D levels and the prevalence of severe left main/trivessel coronary artery disease (CAD), Figure 1B, (lower graph), *= p<0.05 for comparison of I vs II tertile, **= p<0.05 for I vs III tertile.

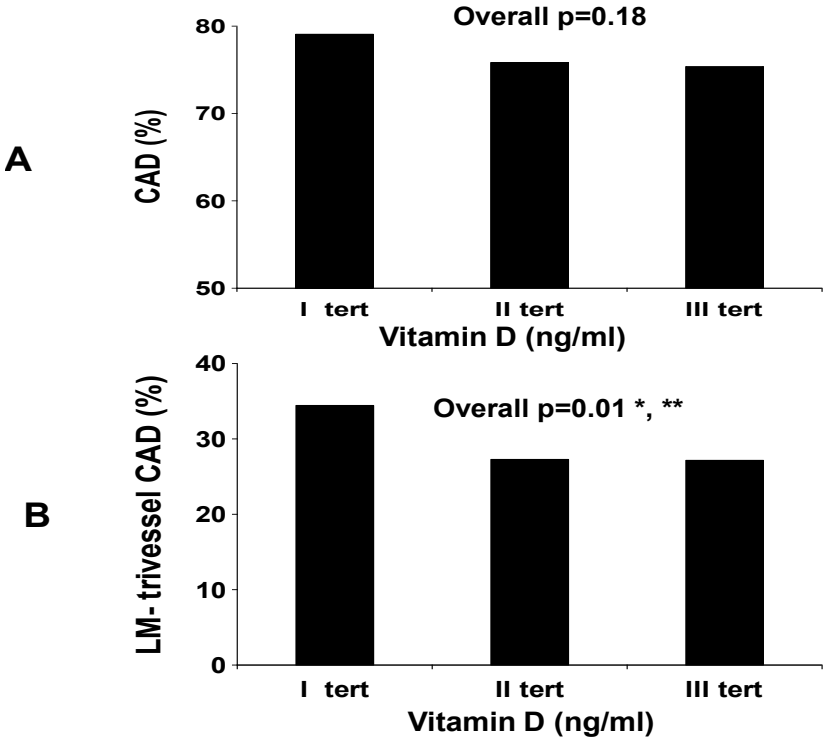
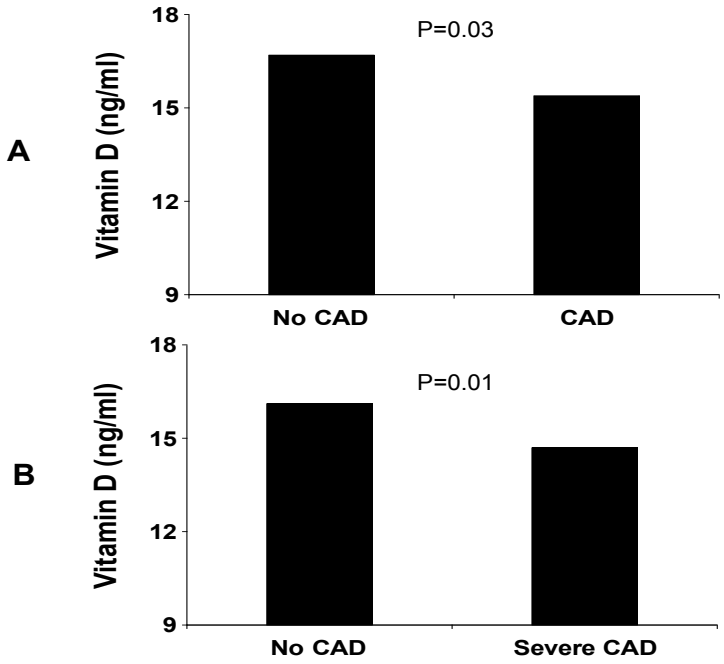


Figure 2. Bar graph showing vitamin D levels in patients with or without coronary disease (2A, upper graph) and severe left main and/or trivessel coronary artery disease (2B, lower graph).



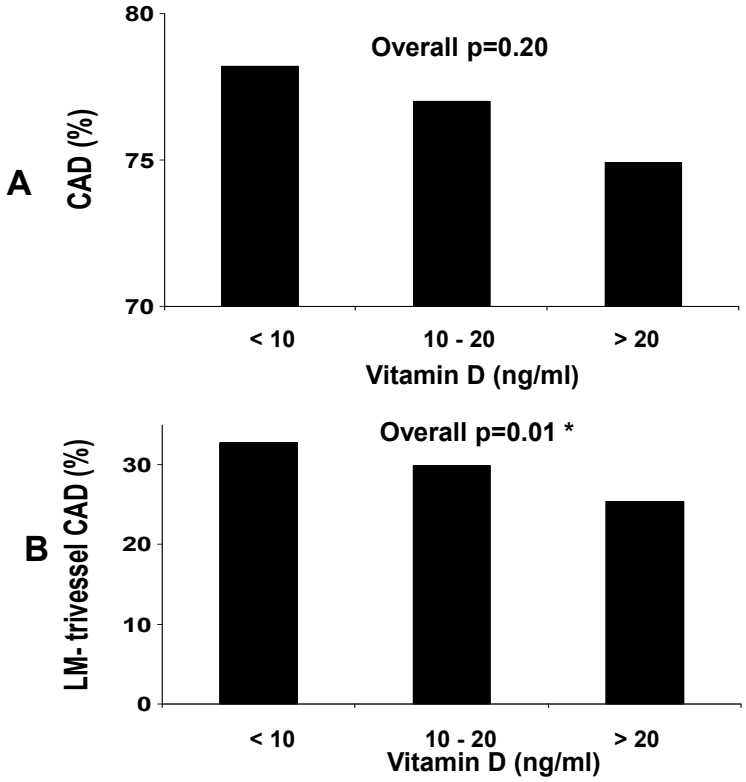

After adjustment for baseline characteristics (age, gender, renal failure, smoking, acute presentation, calcium-antagonists, diuretics, beta-blockers, statins, haemoglobin, platelet count, total and LDL cholesterol, triglycerides), the association between low vitamin D levels and CAD reached a statistical significance (adjusted OR [95%CI] = 1.32[1.1-1.6], p = 0.004), that was confirmed also for severe CAD (adjusted OR [95%CI] = 1.18 [1-1.39], p = 0.05). We additionally analyzed the association between CAD and Vitamin D as continuous variable. As shown in Figure 2, vitamin D levels were significantly lower in CAD patients (15.4± 9.2 vs 16.7±12 ng/mL, p=0.03, Figure 2A) and patients with severe CAD (14.7± 9.2 vs 16.1±10.2, p= 0.01, Figure 2B).

Moreover, the results were confirmed when comparing the extent of coronary atherosclerosis with the severity of vitamin D deficiency (severe deficiency- < 10 ng/ml, n = 510; mild to moderate deficiency -10 to 20 ng/ml-, n = 535). In fact, patients with severe hypovitaminosis showed a non-significant higher prevalence of coronary artery disease (p=0.20, Figure 3A). At multivariate analysis, comparing hypovitaminosis D patients to patients with normal vitamin D status, although no significant association was found between mild hypovitaminosis D and coronary artery disease

(adjusted OR[95%CI]=1.2[0.83-1.72], p=0.34), a statistically significant association was identified between severe hypovitaminosis D and prevalence of coronary artery disease (adjusted OR[95%CI]=1.73[1.18-2.52], p=0.005).

As shown in Figure 3B, a significant relationship was observed between Vitamin D levels and severe CAD. However, only severe Hypovitaminosis D (adjusted OR[95%CI]=1.37[1.001-1.89], p=0.05), but not mild-to-moderate hypovitaminosis D (OR[95%CI]=1.28[0.93-1.76], p=0.13) showed a significantly higher rate of CAD as compared to patients with normal Vitamin D status.

Figure 3. Bar graphs showing the relationship between the severity of vitamin D deficit and the prevalence of coronary artery disease (CAD) Figure 3A (upper graph), and between hypovitaminosis D severity and the prevalence of severe left main/3-vessel coronary artery disease (CAD), Figure 3B, (lower graph), *= p<0.05 for severe deficiency vs normal values.




## Discussion

The main finding of the present study is a significant association between 25-OH D3 and the prevalence of severe coronary artery disease. Furthermore, patients with at least 1 coronary stenosis more than 50% had significantly lower vitamin D status than patients undergoing coronary angiography who did not had a significant coronary artery disease.

Despite the great improvement of revascularization techniques and antithrombotic therapies for the treatment of CAD the results are still unsatisfactory in high-risk subgroups of patients (19). Therefore, great efforts have been done in the last years in order to identify potential new risk factors to prevent and improve outcome of patients with CAD.

Vitamin D is a kind of fat-soluble pro-hormone, that can be introduced both with diet or produced in the skin through the effect of sun UV rays. It is transformed into active form by a first hepatic hydroxylation in 25-position (25-OH D3) and a successive 1-position renal hydroxylation; 25-OH D3 has a 2-3 weeks half-life and its concentration is dependent on levels of new produced hormone. For several reasons much interest has been focused in the last decade on its involvement in the atherosclerotic process. In fact, vitamin D replacement therapy is a very cheap therapeutic strategy (20). Furthermore, Vitamin D deficiency is a rising, widespread endemic problem, especially in Western counties, due to ageing of the population, with decreasing capability of 25-OH D3 synthesis (21), and reduced outdoors living (22), as lower levels of vitamin D have been reported in the elderly, in female gender and in patients with renal failure (234), that are well-established subgroups of patients at high cardiovascular risk. Moreover, hypovitaminosis D has been recently linked to the etiology of hypertension and to a multitude of adverse cardiovascular outcomes (4,6,10), as vitamin D has been reported to modulate endothelial function, inflammatory processes, (244-27), and potentially thrombotic events (28). However, the involvement of vitamin D in the development of atherosclerosis is still debated.

Serum levels of vitamin D have been shown to be inversely associated with the extent of vascular calcifications (29-31) and with carotid intima-media thickness (32), which represent early markers of atherosclerosis. In the large Multi-Ethnic Study of Atherosclerosis (MESA), including 6436 patients with CAD, followed for over 8 years, lower serum 25-OH D3 concentration was associated with an increased risk of incident cardiovascular events among white race participants (33). In addition,

Shanker et al. reported in 287 patients with CAD, vitamin D levels to be significantly lower than in matched healthy controls, with patients in the first vitamin D quartile having a 2.54 times greater risk for CAD than those in the fourth quartile (34).

More recently, Goleniewska et al. evaluating 130 patients with ST-elevation myocardial infarction, identified vitamin D levels as independent predictors of multivessel CAD at multiple stepwise logistic regression (35).

However, data from the combined National Health and Nutrition Examination Survey (NHANES) 2001 to 2006, a population-based cross-sectional study, and the NHANES III cohort, a population-based cohort study, showed that the associations of serum 25(OH)D with mortality, particularly due to cardiovascular disease (CVD) and colorectal cancer, were modified by magnesium intake. In fact, the inverse associations were primarily present among those with magnesium intake above the median (36).

To the best of our knowledge, this represents the first large study investigating the relationship between vitamin D levels and the extent of coronary artery disease as evaluated by coronary angiography. In our population we confirmed the clinical relevance of hypovitaminosis D, that was present in the majority of patients, with seasonal variations form modest to severe deficiency, although still remaining in the range of hypovitaminosis D.

In our study, we identified a significant association between low levels of 25-OH D3 and major risk factors for CAD such as age, female gender, renal failure, hypercholesterolemia, anaemia and smoking, which certainly played a relevant role on our results. In fact, the association between vitamin D deficiency and the extent of CAD resulted statistically significant only after correction for baseline differences. Moreover, a continuous effect of decreasing vitamin D levels and coronary disease can be suggested from our results, as more severe CAD (including left main disease) was

found especially in presence of severe D hypovitaminosis, thus suggesting that patients with vitamin D values < 10 ng/ml should be regarded as those at higher potential cardiovascular risk.

However, no difference was found in terms of angiographic features, like the presence of calcified lesions or intracoronary thrombus, thus suggesting multiple vascular effects of vitamin D, not being mediated just by one preferred pathway. Despite this clinical evidence, contrasting results have been reported so far with cholecalciferol supplementation. In a randomized trial Witham et al (37) found that one single high-dose load of vitamin D2 improved endothelial function, defined as flow-mediated dilatation of the brachial artery. These beneficial effects have been confirmed by Sugden et al. (38) who demonstrated endothelial function improvement in diabetics after vitamin D administration. However, Sokol et al (39) failed to confirm any change in proinflammatory cytokine levels and endothelial function after cholecalciferol repletion in 90 CAD patients. Finally, a recent systematic review and meta-analysis was unable to demonstrate a statistically significant reduction in mortality and cardiovascular risk, including stroke and myocardial infarction, with vitamin D supplementation (40). However, most of these trials were not focused on cardiovascular prevention and did not select patients at higher risk of cardiovascular events, but were mostly focused on the prevention of bone-fracture complications. Therefore, future dedicated larger trials are certainly needed to further investigate the effects of vitamin D supplementation on cardiovascular prevention and atherosclerotic disease progression in high-risk patients.

## LIMITATIONS

The present study was observational. Thus, causal relationship could only be postulated, but not proved. Furthermore, no information on long-term effects of vitamin D levels can be derived from this study, as we did not collect follow-up data. However, previous studies evaluating fetal and early

life vitamin D deficiency revealed an impact on vulnerability to cardiovascular disease in adulthood at a very extended follow-up (41).

Diet, as much as the differences in pharmacological therapy at admission, could have influenced our results, as patients with lower levels of vitamin D were receiving less often statins and beta-blockers, thus potentially increasing their cardiovascular risk. However, our findings were confirmed even at multivariate analysis after correction for potential baseline confounders.

Moreover, the present data are derived from only one region of Northern Italy, and from patients included at only a single academic hospital. Therefore, the results might not be automatically generalized to other populations. In fact, larger vitamin D levels variations could have been identified if the study had been conducted in a different geographical region, allowing more extended sun exposure.

Some results did not reach statistical significance. This could simply reflect the reality or, alternatively, be due to study limitations, such as the classification in tertiles, a relatively small sample size or the presence of several confounding factors. Furthermore, non-calciotropic protective effects on atheroscelerosis may be potentially observed at higher concentrations as compared to calciotropic effects. Finally, there might be some additional factors that we did not explore, such as Vitamin D receptor polymorphisms and levels of Vitamin D binding protein (42).

The use of intravascular ultrasound would have probably improved the results of the current study, as it may provide more accurate information on the amount of coronary atherosclerotic plaque and extent of coronary atherosclerosis, as compared to coronary angiography. In addition, since the aim of the study was to evaluate the extent of coronary artery disease, in order to avoid a potential selection bias, patients undergoing angiography for valvular disease or dilated cardiomyopathy as primary indication were not excluded, due to the possible presence of a concomitant coronary artery disease. We were unable to explain whether Vitamin D could play a role in the development of a

site-specific coronary atherosclerosis, and in particular the higher prevalence of right coronary artery disease observed in patients with low vitamin D levels, that may potentially be due to a play of chance.

## CONCLUSIONS

In patients undergoing coronary angiography severe hypovitaminosis D is frequent, being found among most of the investigated subjects. Furthermore, seasonal variation of vitamin D levels was found. Vitamin D deficiency is significantly associated with the severity of coronary artery disease, defined as left main and/or trivessel disease. Furthermore, patients with significant coronary artery disease had significantly lower vitamin D status than patients who did not show significant stenosis at angiography. The association was stronger for patients with vitamin D levels <10ng/mL. Therefore additional studies are needed to evaluate the potential benefits of vitamin D supplementation on the prevention of CAD and its progression.

## References


1. De Luca G, Cassetti E, Marino P. Percutaneous coronary intervention-related time delay, patient's risk profile, and survival benefits of primary angioplasty vs lytic therapy in ST-segment elevation myocardial infarction. Am J Emerg Med 2009; 27: 712-9.
2. De Luca G, Bellandi F, Huber K, Noc M, Petronio AS, Arntz HR, Maioli M, Gabriel HM, Zorman S, DE Carlo M, Rakowski T, Gyongyosi M, Dudek D.Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab long-term results (EGYPT-ALT) cooperation: individual patient's data meta-analysis. J Thromb Haemost 2011; 9: 2361-70
3. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, Coppo L, Iorio S, Venegoni L, Rondano E, Dell'Era G, Rizzo C, Pergolini P, Monaco F, Bellomo G, Marino P. Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis 2009; 206: 292-7
4. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study. Arch Intern Med. 2008; 168:1174- 1180
5. Ginde AA, Scragg R, Schwartz RS, Camargo CA Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. J Am Geriatr Soc. 2009;57:1595-603.
6. Pilz S, Marz W, Wellnitz B, Seelhorst U, Fahrleitner-Pammer A, Dimai HP, Boehm BO, Dobnig H. Association of vitamin D deficiency with heart failure and sudden cardiac death in a large cross-sectional study of patients referred for coronary angiography. J Clin Endocrinol Metab. 2008; 93:3927-3935
7. Buitrago CG, Arango NS, Boland RL. 1α,25(OH)2D3-dependent modulation of Akt in proliferating and differentiating C2C12 skeletal muscle cells. J Cell Biochem. 2012;113:1170-81
8. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol. 2005;97:93-101
9. Silvagno F, De Vivo E, Attanasio A, Gallo V, Mazzucco G, Pescarmona G. Mitochondrial localization of vitamin D receptor in human platelets and differentiated megakaryocytes. PLoS One. 2010 ;5:e8670
10. Forman JP, Bischoff-Ferrari HA, Willett WC, Stampfer MJ, Curhan GC. Vitamin D intake and risk of incident hypertension: results from three large prospective cohort studies. Hypertension. 2005; 46:676-682.
11. Pittas AG, Lau J, Hu FB, Dawson-Hughes B. The role of vitamin D and calcium in type 2 diabetes. A systematic review and meta-analysis. J Clin Endocrinol Metab. 2007;92:2017-2029.
12. Lamendola CA, Ariel D, Feldman D, Reaven GM. Relations between obesity, insulin resistance, and 25-hydroxyvitamin D. Am J Clin Nutr. 2012; 95:1055-105
13. Ford ES, Ajani UA, McGuire LC, Liu S. Concentrations of serum vitamin D and the metabolic syndrome among U.S. adults. Diabetes Care. 2005; 28:1228-1230.
14. Mancia G, Fagard R, Narkiewicz K, Redón J, Zanchetti A, Böhm M, et al. 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J Hypertens. 2013;31:1281-357
15. Holick MF. Vitamin D deficiency. N Engl J Med. 2007 Jul 19;357(3):266-81

16. Vierucci F, Del Pistoia M, Fanos M, Gori M, Carlone G, Erba P, Massimetti G, Federico G, Saggese G. Vitamin D status and predictors of hypovitaminosis D in Italian children and adolescents: a cross-sectional study. Eur J Pediatr. 2013 Dec;172(12):1607-17
17. Yong AS, Ng AC, Brieger D, Lowe HC, Ng MK, Kritharides L. Three-dimensional and two-dimensional quantitative coronary angiography, and their prediction of reduced fractional flow reserve. Eur Heart J. 2011 Feb;32(3):345-53.
18. De Luca G, van 't Hof AW, Ottervanger JP, Hoorntje JC, Gosselink AT, Dambrink JH, de Boer MJ, Suryapranata H. Ageing, impaired myocardial perfusion, and mortality in patients with ST-segment elevation myocardial infarction treated by primary angioplasty. Eur Heart J 2005; 26: 662-6.
19. De Luca G, Ernst N, van 't Hof AW, Ottervanger JP, Hoorntje JC, Gosselink AT, Dambrink JH, de Boer MJ, Suryapranata H. Predictors and clinical implications of early reinfarction after primary angioplasty for ST-segment elevation myocardial infarction. Am Heart J. 2006; 151: 1256-9
20. Grant WB, Cross HS, Garland CF, Gorham ED, Moan J, Peterlik M, Porojnicu AC,Reichrath J, Zittermann A. Estimated benefit of increased vitamin D status in reducing the economic burden of disease in western Europe. Prog Biophys Mol Biol. 2009; 99(2-3):104-13
21. Holick, M. F., Matsuoka, L. Y., Wortsman, J. (1989). Age, vitamin D, and solar ultraviolet. The Lancet 1989; 2: 1104–1105.
22. Holick, M. F., MacLaughlin, J. A., Doppelt, S. H. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. Science, 211, 590–593.
23. Rostand SG, Drueke TB. Parathyroid hormone, vitamin D, and cardiovascular disease in chronic renal failure. *Kidney Int* 1999;56:383–39
24. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, Toprak A, Yazici D, Sancak S, Deyneli O, Akalin S. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. *J Clin Endocrinol Metab* 2009;94:4023–4030.
25. Cardus A, Panizo S, Encinas M, Dolcet X, Gallego C, Aldea M, Fernandez E, Valdivielso JM. 1,25-dihydroxyvitamin D3 regulates VEGF production through a vitamin D response element in the VEGF promoter. *Atherosclerosis* 2009;204:85–89.
26. Norman PE, Powell JT. Vitamin D, shedding light on the development of disease in peripheral arteries. *Arterioscler Thromb Vasc Biol*. 2005;25:39–46
27. *S*chleithoff SS, Zittermann A, Tenderich G, Berthold HK, Stehle P, Koerfer R. Vitamin D supplementation improves cytokine profiles in patients with congestive heart failure: A double-blind, randomized, placebo-controlled trial. *Am J Clin Nutr* 2006;83:754–759
28. *A*ihara K, Azuma H, Akaike M, Ikeda Y, Yamashita M, Sudo T, Hayashi H, Yamada Y, Endoh F, Fujimura M, Yoshida T, Yamaguchi H, Hashizume S, Kato M, Yoshimura K, Yamamoto Y, Kato S, Matsumoto T. Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice. J Biol Chem. 2004; 279:35798-35802
29. Sigmund CD. Regulation of renin expression and blood pressure by vitamin D(3). *J Clin Invest* 2002;110:155–156
30. Barreto DV, Barreto FC, Liabeuf S, Temmar M, Boitte F, Choukroun G, Fournier A, Massy ZA. Vitamin D affects survival independently of vascular calcification in chronic kidney disease. *Clin J Am Soc Nephrol*. 2009;4:1128–1135.
31. Jono S, Nishizawa Y, Shioi A, Morii H. 1,25-Dihydroxyvitamin D3 increases in vitro vascular calcification by modulating secretion of endogenous parathyroid hormone-related peptide. *Circulation*. 1998; 98:1302–1306.
32. Watson KE, Abrolat ML, Malone LL, Hoeg JM, Doherty T, Detrano R, Demer LL. Active serum vitamin D levels are inversely correlated with coronary calcification. Circulation.1997 16; 96:1755-1760
33. *R*obinson-Cohen C, Hoofnagle AN, Ix JH, Sachs MC, Tracy RP, Siscovick DS, Kestenbaum BR, de Boer IH. Racial differences in the association of serum 25-hydroxyvitamin D concentration with coronary heart disease events. JAMA 2013; 310: 179-88
34. Shanker J, Maitra A, Arvind P, Nair J, Dash D, Manchiganti R, Rao VS, Radhika KN, Hebbagodi S, Kakkar VV. Role of vitamin D levels and vitamin D receptor polymorphisms in relation to coronary artery disease: the Indian atherosclerosis research study. Coron Artery Dis 2011; 22: 324-32.
35. Goleniewska BM, Kacprzak M, Zielinska M. Vitamin D level and extent of coronary stenotic lesions in patients with first acute myocardial infarction. Cardiol J 2013 Jun 25 [Epub ahead of print].
36. Deng Deng X, Song Y, Manson JE, Signorello LB, Zhang SM, Shrubsole MJ, Ness RM, Seidner DL, Dai Q. Magnesium, vitamin D status and mortality: results from US National Health and Nutrition Examination Survey (NHANES) 2001 to 2006 and NHANES III. BMC Med 2013 Aug 27 [Epub ahead of print].
37. Witham MD, Dove FJ, Sugden JA, Doney AS, Struthers AD. The effect of vitamin D replacement on markers of vascular health in stroke patients – A randomised controlled trial. Nutr Metab Cardiovasc Dis 2012; 22: 864-70.
38. Sugden JA, Davies JI, Witham MD, Morris AD, Struthers AD. Vitamin D improves endothelial function in patients with type 2 diabetes mellitus and low vitamin D levels. Diab Med 2008; 25: 320–325
39. Sokol SI, Srinivas V, Crandall JP, Kim M, Tellides G, Lebastchi AH, Yu Y, Gupta AK, Alderman MH. The effects of vitamin D repletion on endothelial function and inflammation in patients with coronary artery disease. Vasc Med. 2012; 17: 394-404.
40. Elamin MB, Abu Elnour NO, Elamin KB, Fatourechi MM, Alkatib AA, Almandoz JP, Liu H, Lane MA, Mullan RJ, Hazem A, Erwin PJ, Hensrud DD, Murad MH, Montori VM. Vitamin D and cardiovascular outcomes: a systematic review and meta-analysis. J Clin Endocrinol Metab 2011; 96: 1931-42.
41. Gezmish O, Black MJ. Vitamin d deficiency in early life and the potential programming of cardiovascular disease in adulthood. J Cardiovasc Transl Res 2013; 6: 588-603.
42. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, Powe NR, Thadhani R. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013;369(21):1991-2000

# Chapter 3:

## Impact of high-dose statins on vitamin D levels and platelet function in patients with coronary artery disease

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### Abstract

**Background.** Statins represent a pivotal treatment in coronary artery disease, offering a reduction in cardiovascular risk even beyond their lipid-lowering action. However, the mechanism of these "pleiotropic" benefits of statins are poorly understood. Vitamin D has been suggested as a potential mediator of the anti-inflammatory, anti-thrombotic and vascular protecting effects of statins. Aim of present study was to assess the impact of a high-intensity statin therapy on vitamin D levels and platelet function in patients with coronary artery disease.

**Methods.** Patients discharged on dual antiplatelet therapy and high-intensity statins after an ACS or elective PCI were scheduled for main chemistry and vitamin D levels assessment at 30-90 days post-discharge. Vitamin D (25-OHD) dosing was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). Platelet function was assessed by Multiplate® (multiple platelet function analyser; Roche Diagnostics AG).

**Results**. Among 246 patients included, 142 were discharged on a new statin therapy or with an increase in previous dose (Inc-S), while 104 were already receiving a high-dose statin at admission, that remained unchanged (eq-S). Median follow-up was 75.5 days. Patients in the Inc-S group were younger (p=0.01), smokers (p<0.001), with a lower history of hypercholesterolemia (p=0.05), diabetes (p=0.03), hypertension (p=0.02), or previous cardiovascular events (p<0.001). They were

more often admitted for an acute coronary syndrome (p<0.001) and used less frequently anti-hypertensive drugs or nitrates. Higher total circulating calcium was observed in the Inc-S group (p=0.004), while baseline vitamin D levels were similar in the 2 groups (p=0.30). A significant reduction in the circulating low-density lipoprotein (LDL) cholesterol was observed in the Inc-S group. Vitamin D levels increased in the Inc-S patients but not in the eq-S group (delta-25OHD: 23.2±20.5% vs 3.1±4.7%, p=0.003), with a linear relationship between the magnitude of vitamin D elevation and the reduction of LDL cholesterol (r=-0.17, p=0.01). Platelet reactivity was significantly lower in the Inc-S patients, when evaluating aggregation with different platelet activating stimuli (arachidonic acid, p=0.02, collagen, p=0.004, thrombin-activating peptide, p=0.07, ADP, p=0.002).

Conclusions. In patients with coronary artery disease, the addition of a high-intensity statin treatment, besides the lipid-lowering effects, is associated to a significant increase in vitamin D levels and lower platelet reactivity, potentially providing explanation of the “pleiotropic” benefits of statins therapy in cardiovascular disease.

Keywords: vitamin D, statins; platelet aggregation, coronary artery disease

## Introduction

In the era of interventional cardiology representing the first treatment option for the majority of patients with coronary artery disease (CAD), a crucial role in cardiovascular prevention is still played by pharmacological therapy (1-3). Statins, the inhibitors of cholesterol synthesis, are strongly recommended in all patients with coronary artery disease, having demonstrated in several trials a 25-40% reduction in cardiovascular risk (4), directly related to the magnitude of low-density lipoprotein cholesterol (LDL-C) lowering (5). Moreover, a relevant regression in the volume of atheromasic plaque has been demonstrated with high-intensity statins, those achieving over 40% cholesterol reduction (6,7). However, the benefits of this therapy in CAD has been ascribed not only to an aggressive reduction of circulating pro-atherogenic lipid particles, but also to its "pleiotropic" effects, including improvement in endothelial function, anti-inflammatory and anti-oxidant actions, whose mechanism is still largely undefined (8,9).

Previous reports have suggested a role of vitamin D in explaining the pleiotropic effects of statins (10,11). In fact, vitamin D (25-OHD) is the precursor of a hormone with a widespread cardio-protective function, modulating inflammatory and thrombotic processes (12). Hypovitaminosis D, indeed, has been linked to the development of main established cardiovascular risk factors, as hypertension or diabetes, and to an increased risk of CAD and acute myocardial infarction (13-15). However, despite, in certain studies, statin administration could raise the circulating levels of 25-OHD in patients with dyslipidaemia or diabetes (16,17), confirming a potential vitamin D-statin interplay in cardiovascular prevention, results were not confirmed by other reports (18), with even few data in patients with established CAD.

Therefore, aim of present study was to evaluate the impact of a high-intensity statin therapy on vitamin D levels and platelet function in patients with a recent acute coronary syndrome or percutaneous coronary interventions.

## Methods

We included patients admitted Division of Cardiology, “Maggiore della Carità” Hospital, Università del Piemonte Orientale in Novara, Italy, from September 2013 to December 2014 requiring dual antiplatelet therapy for acute coronary syndromes or after PCI for stable coronary artery disease. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criterion. All patients receiving at discharge high-intensity statins and a dual antiplatelet therapy with ASA (100 to 160 mg daily) and ADP-antagonist (clopidogrel 75 mg daily or ticagrelor 90 mg b.i.d) were scheduled for chemistry and platelet function tests evaluation at 30-90 days from discharge.

High-intensity statin therapy was considered for atorvastatin ≥ 20 mg/daily, rosuvastatin ≥ 10 mg/daily or simvastatin ≥ 40 mg daily according to literature (19).

The study was approved by our local Ethical Committee and informed consent was obtained by all patients. The study was conducted in accordance with the Declaration of Helsinki.

Main demographic, clinical and angiographic data, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. Patients receiving concomitant vitamin D supplementation were excluded. As previously described (20) hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg or if the individual was taking antihypertensive medications. Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycemia > 126 mg/dL or HbA1c > 6.5%. Chronic renal failure was considered for a history of renal failure or an admission glomerular filtrate (GFR) < 60 mol/min/1.73m2 by MDRD (Modification of Diet in renal Disease) formula.

## Biochemical measurements

Fasting blood samples were drawn from all patients for main chemistry and vitamin D levels assessment at baseline, at admission and at planned follow-up. Vitamin D dosing was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for 25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference (21).

## Platelet function assessment

Platelet aggregation was measured by a whole blood test, the impedance aggregometry (Multiplate®-multiple platelet function analyser; Roche Diagnostics AG) For Multiplate a whole blood sample was stored in Vacutainer standard lithium heparin tubes and analyzed within 1-2 hours from collection (22). Tests with different agonists were performed: arachidonic acid (AA), collagen, ADP and prostaglandin E1 and thrombin receptor activating peptide (TRAP-6). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min).

## Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data were expressed as mean $\pm$ SD and categorical data as percentage. Patients were divided in 2 groups: in the "eq-S" group we included patients already receiving at admission a high-intensity statin, that was maintained on discharge, while the "Inc-S" were considered patients who started a high-intensity statin therapy or increased the dose or a previous treatment on discharge. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Linear regression analysis was performed to evaluate the relationship between changes in vitamin D levels and the reduction in low-density lipoprotein (LDL) cholesterol. The Mann-Withney U test was used to compare median follow-up values. The changes in vitamin D and LDL cholesterol levels were

considered both as an absolute difference or as % variation, as compared to baseline. A p value < 0.05 was considered statistically significant.

Table 1. Clinical characteristics according to statins therapy dosage.

| Baseline clinical characteristics | Inc-Statin therapy N= 142 | eq-Statin therapy N= 104 | P value |
|---|---|---|---|
| Age (mean±SD) | 63.9±11.6 | 67.2±8.8 | 0.01 |
| Ag ≥75 years (%) | 21.7 | 21.2 | 0.99 |
| Male Sex (%) | 75.9 | 85 | 0.29 |
| BMI (mean±SD) | 27.5±4.7 | 27.9±4.8 | 0.41 |
| Hypercholesterolemia(%) | 57.4 | 69 | 0.05 |
| Diabetes mellitus (%) | 39.5 | 52.2 | 0.03 |
| Renal failure (%) | 12.7 | 16.8 | 0.40 |
| Active smokers (%) | 36 | 14.2 | <0.001 |
| Hypertension (%) | 69.8 | 82.3 | 0.02 |
| History of MI (%) | 13.3 | 34.5 | <0.001 |
| Previous PCI (%) | 26.5 | 62.8 | <0.001 |
| Previous CABG (%) | 7.9 | 13.3 | 0.16 |
| Indication to angiography | | | <0.001 |
| Stable angina/ silent ischemia (%) | 21.6 | 54.9 | |
| STEMI/ACS (%) | 74.7 | 41.6 | |
| Other (%) | 3.7 | 3.5 | |
| Severe left main/trivessel CAD (%) | 24.7 | 41.1 | 0.005 |
| Hypovitaminosis D (%) | 22.3 | 23.4 | 0.87 |
| Concomitant medications (at follow-up) | | | |
| ACE inhibitors(%) | 59.5 | 48.7 | 0.07 |
| ARB (%) | 17.4 | 29.2 | 0.02 |
| Beta blockers (%) | 84.2 | 82.3 | 0.75 |
| Nitrates (%) | 40 | 52.2 | 0.04 |
| Calcium antagonists (%) | 16.8 | 32.7 | 0.002 |
| Diuretics (%) | 21.6 | 32.7 | 0.04 |
| Ticagrelor(%) | 78.9 | 56.7 | 0.06 |

*CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers*;

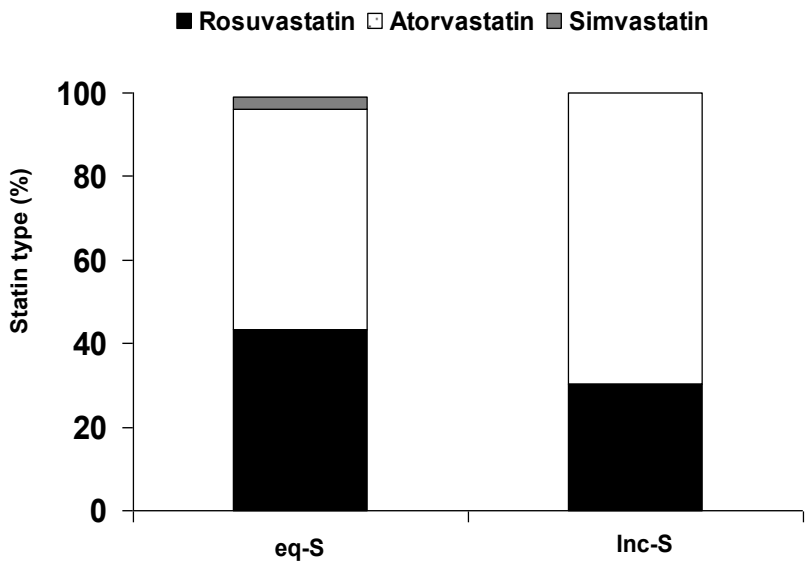
## Results

Our population is represented by a total of 246 patients. Among them, 142 patients were discharged on a new statin therapy or with an increase in previous dose (Inc-S), while 104 were receiving at admission a high-intensity statin, that remained unchanged (eq-S). As shown in Figure 1, in the Inc-S group 69.8% of patients received atorvastatin and 30.2% rosuvastatin, while in the eq-S group, 52.9%

of patients received atorvastatin, 43.3% rosuvastatin and 2.8% simvastatin. Median follow-up was 75.5 days [Interquartile Range(IQR): 38-84.8]; not being different in the eq-S group (median: 65.5 days; IQR[42-83]) or the Inc-S group (median 79 days; IQR[41-87], p value =0.19).

Table 1 shows main demographic and clinical features of the study population. Patients in the Inc-S group were younger (p=0.01), smokers (p<0.001), with a lower history of hypercholesterolemia (p=0.05), diabetes (p=0.03), hypertension (p=0.02), or previous cardiovascular events (p<0.001). They were more often admitted for acute coronary syndrome (p<0.001) and used less frequently anti-hypertensive drugs or nitrates. No difference in main chemistry parameters was observed, as displayed in Table 2, but for higher total circulating calcium in the Inc-S group (p=0.004). Also baseline vitamin D levels were similar in the 2 groups (p=0.30), while mean values of vitamin D at re-assessment displayed a non significant trend for higher values in the Inc-S group (p=0.09).

Figure 1 Distribution of statin therapy in study population (eq-S= statin dose unchanged, Inc-S= statin dose increased or new therapy started)




Platelet reactivity was significantly lower in the Inc-S patients, when evaluating aggregation with different platelet activating stimuli (arachidonic acid, p=0.02, collagen, p=0.004, thrombin-activating peptide, p=0.07, ADP, p=0.002), Table 2.

Table 2. Biochemistry parameters at follow-up according to statin therapy dosage

| Biochemistry parameters [α] | Inc-Statin therapy N= 142 | eq-Statin therapy N= 104 | P value |
|---|---|---|---|
| Platelets (10^5/µl; mean± SD) | 232.8±72.2 | 229.4±65.2 | 0.68 |
| Haemoglobin (g/dl± SD) | 13.7±1.7 | 13.7±1,6 | 0.92 |
| WBC (10^3/µl;mean± SD) | 8.5±6.6 | 7.9±1.9 | 0.45 |
| HDL cholesterol (mg/dL± SD) | 41.3±12.2 | 40.9±11.2 | 0.76 |
| LDL cholesterol (mg/dl± SD) | 69.8±24.1 | 70.5±25.7 | 0.82 |
| Triglycerides (mg/dl±SD) | 121.5±80.5 | 133.2±66 | 0.20 |
| Fibrinogen (mg/dl± SD) | 402±123.9 | 379.7±114.3 | 0.12 |
| Glycaemia (mg/dL± SD) | 124.3±57.2 | 126.9±47.4 | 0.68 |
| Glycosylated haemoglobin (%± SD) | 6.4±1.2 | 6.7±1.3 | 0.12 |
| Creatinine (mg/dL± SD) | 0.9±0.5 | 1±0.4 | 0.32 |
| C reactive protein (mg/dL±SD) | 0.5±1.2 | 0.3±0.5 | 0.13 |
| Calcium (mg/dL±SD) | 9.1±0.42 | 8.8±1.1 | 0.004 |
| Parathyroid hormone –active (mg/dL±SD) | 65.9±47.5 | 69.1±49.5 | 0.61 |
| Vitamin D – follow-up (ng/ml±SD) | 17.9±9.8 | 16.1±8 | 0.09 |
| Vitamin D – baseline (ng/ml±SD) | 15.5±8.7 | 16.7±8.2 | 0.30 |
| ASPI test (AU*min± SD) | 344±185.7 | 398.7±208.1 | 0.02 |
| COL test (AU*min± SD) | 418.9±141.7 | 469.3±152.7 | 0.004 |
| TRAP test (AU*min± SD) | 1070.2±266.8 | 1132.2±306.8 | 0.07 |
| ADP test (AU*min± SD) | 307.6±172.1 | 374.3±181.9 | 0.002 |

[α] mean values of each parameter are reported for dosing at follow-up but for baseline levels of vitamin D.

A significant reduction in circulating low-density lipoprotein (LDL) cholesterol was observed in the Inc-S group, and not in the eq-S patients, when evaluating both absolute difference (-45.2±40.6 mg/dl vs -4.5±29.9 mg/dl, p<0.001) and the percentage of LDL reduction (-33.5±29.8 % vs -1.8±41%, p<0.001; Figure 2). Vitamin D levels increased in the Inc-S patients but not in the eq-S group (delta-25OHD: 2.08±6.6 vs 0.26±7.5 ng/ml, p=0.048). Moreover, the variation was even enhanced among those 127 statin naive- patients at baseline, (delta vitamin D: 2.28±6.7).

Results were similar when evaluating the percentage of 25OHD variation (23.2±20.5% vs 3.1±4.7%, p=0.003), as in Figure 3.

Figure 2 Bar graph showing the difference in low-density lipoprotein (LDL) cholesterol, as delta LDL, after 30-90 days statin therapy in the 2 study groups (eq-S= statin dose unchanged, Inc-S= statin dose increased or new therapy started)

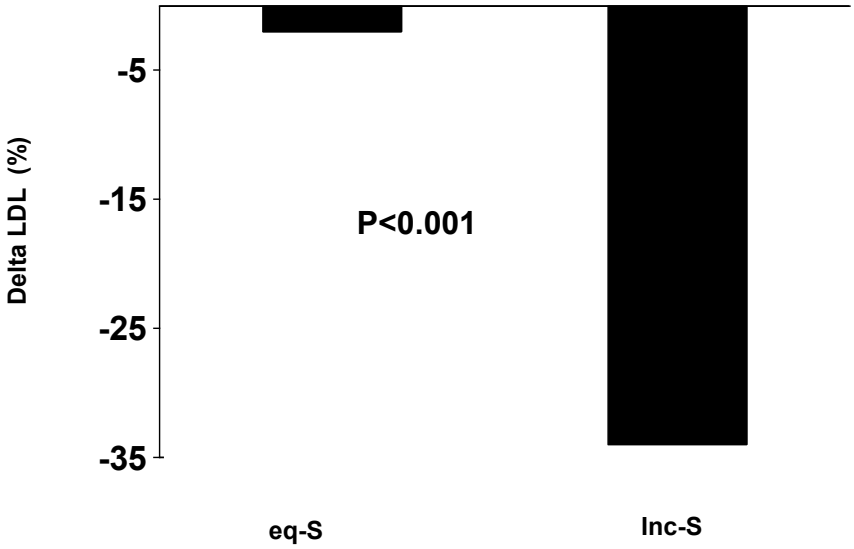
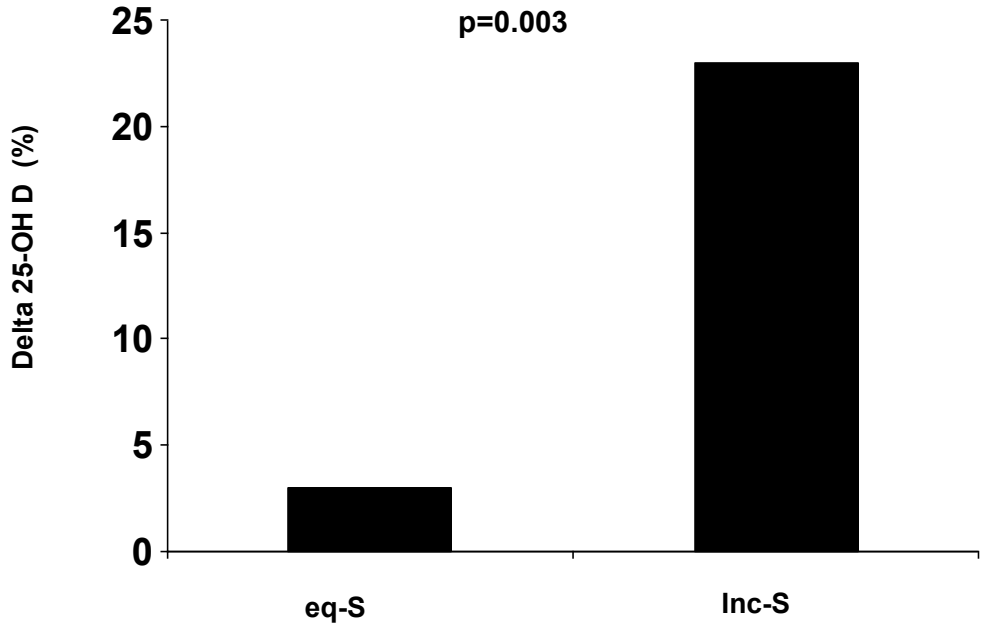
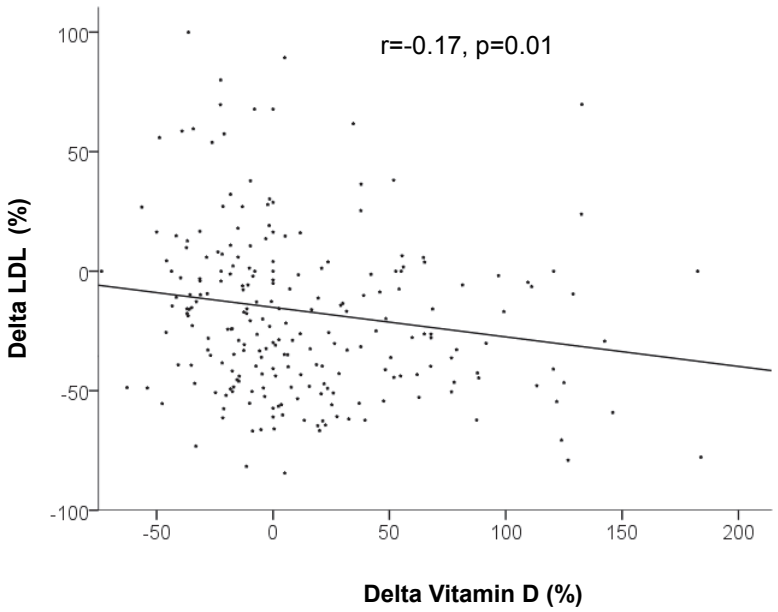



Figure 3 Bar graph showing the difference in vitamin D levels, as delta 25-OH D, after 30-90 days statin therapy in the 2 study groups (eq-S= statin dose unchanged, Inc-S= statin dose increased or new therapy started)




We additionally observed a significant correlation between the lipid-lowering effectiveness of statins and vitamin D levels, as we described a linear relationship between the magnitude of vitamin D elevation and the reduction of LDL cholesterol (r=-0.17, p=0.01), Figure 4.

**Figure 4** Linear regression between the magnitude of low-density lipoprotein (LDL) cholesterol decrease and the difference in vitamin D levels




## Discussion

The present study represents one of the first and largest attempts to define the role of high-intensity statins on vitamin D levels and their impact on platelet function in patients with coronary artery disease (CAD).

Our main finding is a significant elevation, when considering the variation from baseline, of circulating 25-OHD after about 3 months of a new or increased dose- statin therapy, going along with a similar LDL-C reduction, that was not observed in a comparable group of patients chronically and stably treated with statins for coronary artery disease.

Despite significant improvements in antithrombotic therapies and mechanical reperfusion strategies (23-25), the quote of patients experiencing acute cardiovascular events is still raising, thus requiring further efforts in developing measures for cardiovascular prevention (26).

Therefore, particular attention has been focused to those non-canonical cardiovascular risk factors, such as hypovitaminosis D, that could offer innovative pharmacological targets and potential adjunctive benefits in patients with CAD (27).

Vitamin D deficiency, in fact, represents a worldwide health problem, whose social and economic impact is growing, together with the ageing of the population and the increasing complexity and frailty of patients treated for coronary artery disease.

In fact, lower levels of 25-OHD have been inversely associated with vascular calcifications, arterial stiffness, major cardiovascular risk factors and with the extent of coronary artery disease (28,29). However, vitamin D has received, in the last few years, great interests also for its multiple effects on inflammatory system and atherothrombosis.

In fact, its receptor has been identified also on inflammatory cells, where the active metabolites of vitamin D can inhibit leukocytes proliferation and the release of cytokines in the bloodstream (30), and even in platelets, preventing their aggregation (31).

Similar anti-inflammatory, anti-oxidant and antithrombotic properties have been ascribed to statins, representing their non-lipid lowering related “pleiotropic effects (8).

These non-lipid dependent benefits have emerged since the first large studies with statins, as the WOSCOP and CARE trials (32,33), where statin treated patients had a significantly lower cardiovascular risk as compared to non-statin treated patients with similar cholesterol levels.

In fact, in addition to a reduction in the risk of major cardiovascular events of up to 21% for every 1 mmol/l (39 mg/dl) decrease in LDL-C (34), statins have demonstrated to favour the improvement of endothelial dysfunction, increase nitric oxide bioavailability, inhibit inflammatory responses and promote a stabilization of atherosclerotic plaques (9).

However, the underlying mechanism of statins’ “pleiotropic effects” is still poorly understood.

Previous studies have suggested that these beneficial effects of statins could be dependent on an increase of vitamin D levels. In fact, Pérez-Castrillon et al. documented that vitamin D levels significantly increased in patients with acute ischemic heart disease after the treatment with atorvastatin (11). They postulated that the increase in 25-OH D induced by atorvastatin was a

consequence of the inhibition of cholesterol synthesis at the HMG-CoA reductase enzyme, resulting in a raise in the levels of 7-dehydrocholesterol, a precursor of vitamin D. Analogous findings were observed with lovastatin and simvastatin in patients with familial hypercholesterolemia and with atorvastatin in patients with type 2 diabetes mellitus (17,35,36).

In the Statin-D trial (37), 134 hyperlipidemic patients were randomized to fluvastatin or rosuvastatin therapy. After 8 weeks treatment, no change in 25-OH D was observed with fluvastatin, whereas rosuvastatin almost triplicated mean vitamin D levels and significantly decreased the activity of bone alkaline phosphatase. In fact, Staal et al. (38) demonstrated in cell culture experiments, that inhibition of osteoclastic activity was inversely correlated with the magnitude of a HMG-CoA reductase activity of statins, thus suggesting that higher-intensity statins could be more effective in modifying vitamin D levels.

In contrast with these reports, however, Thabit et al. (39) identified no impact of simvastatin and atorvastatin, at any dose, for a duration of more than one year, on 25-OH D levels.

Similar negative results were obtained by Anagnostis et al. in 63 dyslipidemic patients receiving atorvastatin or rosuvastatin for 12 weeks (40).

Therefore, controversy still exists on the interaction of statin and vitamin D, with few data reported so far in subjects with proven CAD. Present study firstly included a population receiving high-intensity statins for secondary prevention after an acute coronary syndrome or PCI. We evaluated the modifications in vitamin D levels in patients with a first prescription of high-dose statin therapy or undergoing a dosage increase (Inc-S) versus those patients chronically and stably treated with statin (unchanged dosage at hospital discharge) (Eq-S).

After 30 to 90 days treatment (median 75 days), vitamin D levels were unvaried in the Eq-S group while they significantly increased in the Inc-S group, with the extent of 25-OH D elevation depending on the magnitude of LDL-lowering effect. However, these acute effects, did not tend to progressively

increase over time, as no difference in vitamin D levels was observed in patients chronically treated with statins.

In addition, the levels of platelet reactivity during dual antiplatelet therapy with ASA and clopidogrel or ticagrelor were significantly lower, with four different aggregating stimuli, in patients achieving a more significant increase in vitamin D levels.

Indeed, the hypothesis of a crosstalk between vitamin D and statins metabolism had already been raised by Schwarz et al. (41), demonstrating that vitamin D supplementation could lower atorvastatin and its metabolites concentrations, while showing synergistic effects on cholesterol concentrations. In fact, CYP3A4 catabolizes vitamin D in liver and intestine (42). Statins are extensively metabolized and display an inhibitory activity on by CYP3A4 and CYP3A5. This common catabolic pathway may be responsible for the increased 25-OH D levels during statin treatment.

In addition, Grimes (43) has suggested that statins might act as vitamin D analogues and compete with vitamin D for its receptor. Although rosuvastatin has not been clearly demonstrated to bind vitamin D receptor, it can target the glucocorticoid receptor and the thyroid beta-1 receptor, both of which interact with vitamin D metabolites.

Therefore, the present data provide further confirmation and a more deep insight in explaining the beneficial role of statins- vitamin D interplay in patients at high cardiovascular risk, with a particular focus on platelet function.

Nevertheless, the exact mechanism of such pharmacological interaction still needs to be elucidated in more dedicated studies, as far as a potential role of vitamin D supplementation in achieving the same cardio-protective effects with a lower dose of statins, improving tolerance and adherence to this drugs. In fact, higher 25-OH D levels have demonstrated to prevent statin-induced myalgia (44).

Limitations.

A first limitation is represented by the cross-sectional design of the study, including a certain degree of heterogeneity in patients' characteristics and statin therapy. However, we deemed that this kind of population, including very high-risk patients, could be more representative of the real world population, therefore providing more helpful information for everyday clinical practice. Indeed, it might be argued that the clinical differences between the two study groups could have influenced the baseline levels of vitamin D. In fact, patients in the Eq-Statin therapy were significantly older, and with higher rates of diabetes, that are know risk factors for vitamin D deficiency. However, this observation further reinforces our hypothesis, as the chronic use of statins in these patients might potentially have counterbalanced their tendency to hypovitaminosis D. In fact, they displayed non-inferior or even slightly higher baseline levels of Vitamin D, as compared to the Inc-Statin arm, even though not statistically significant.

In addition, the sample size would not have allowed us to perform a subgroup analysis or a propensity score matching, that would have implied a further division of each group.

Furthermore, the rate of patients on ASA+clopidogrel or ASA + ticagrelor was not different between the two groups, thus not having represented a potential confounding factor for our data on platelet reactivity. Indeed, the absence of a baseline assessment of platelet function cannot allow to demonstrate the eventual changes in platelet reactivity induced by vitamin D raise, nevertheless, we previously demonstrated the strict relationship between Multiplate tests results and 25-OHD also in patients on DAPT (45).

Moreover, we did not evaluate whether vitamin D supplementation could improve the lipid-lowering effects of statins or the response to antiplatelet agents and we also excluded the few patients already on vitamin D therapy, as different dosing and formulations of the vitamin could have represented a potential bias.

Finally, we did not collect data at long term follow-up, therefore we cannot tell on the impact of vitamin D levels or platelet reactivity on the occurrence of cardiovascular events in our patients. However, previous studies have already demonstrated the prognostic role of platelet reactivity, as defined by Multiplate aggregometry, on the risk of major ischemic events (46).

## Conclusion.

In patients with coronary artery disease, the addition of a high-intensity statin treatment, besides the lipid-lowering effects, is associated to a significant increase in vitamin D levels and to lower platelet reactivity, potentially providing explanation of the "pleiotropic" benefits of statins therapy in cardiovascular disease.

**References**


1. De Luca G, Biondi-Zoccai G, Marino P. Transferring patients with ST-segment elevation myocardial infarction for mechanical reperfusion: a meta-regression analysis of randomized trials. Ann Emerg Med. 2008;52(6):665-76
2. De Luca G, Marino P. Facilitated angioplasty with combo therapy among patients with ST-segment elevation myocardial infarction: a meta-analysis of randomized trials. Am J Emerg Med. 2009;27(6):683-90.
3. Verdoia M, Schaffer A, Suryapranata H, De Luca G. Effects of HDL-modifiers on cardiovascular outcomes: A meta-analysis of randomized trials. Nutr Metab Cardiovasc Dis. 2015;25(1):9-23.
4. Cholesterol Treatment Trialists Collaborators. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet.2005;366(9493):1267–78
5. Ballantyne CM. Low-density lipoproteins and risk for coronary artery disease. Am J Cardiol. 1998;82:3Q–12Q
6. Nissen SE, Nicholls SJ, Sipahi I, Libby P, Raichlen JS, Ballantyne CM, Davignon J, Erbel R, Fruchart JC, Tardif JC, Schoenhagen P, Crowe T, Cain V, Wolski K, Goormastic M, Tuzcu EM; ASTEROID Investigators. Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial. JAMA. 2006;295(13):1556-65
7. Law M R, Wald N J, Rudnicka A R. Quantifying effect of statins on low density lipoprotein cholesterol, ischaemic heart disease, and stroke: systematic review and meta-analysis. BMJ 2003; 326: 1423.
8. Wang CY, Liu PY, Liao JK. Pleiotropic effects of statin therapy: molecular mechanisms and clinical results. Trends Mol Med, 2008; 14: 37–44.
9. Davignon J. Beneficial cardiovascular pleiotropic effects of statins. Circulation. 2004;109(23 Suppl 1):III39-43
10. Pera Ł, Sygitowicz G, Białek S, Łukaszkiewicz J, Sitkiewicz D. Vitamin D and statins: action in preventing cardiovascular events. Kardiol Pol. 2012;70(12):1296-8
11. Perez-Castrillon JL, Vega G, Abad L et al. Effects of atorvastatin on vitamin D levels in patients with acute ischemic heart disease. Am J Cardiol, 2007; 99: 903–905
12. Kunadian V, Ford GA, Bawamia B, Qiu W, Manson JE. Vitamin D deficiency and coronary artery disease: a review of the evidence. Am Heart J. 2014;167(3):283-91
13. Lamendola CA, Ariel D, Feldman D, Reaven GM. Relations between obesity, insulin resistance, and 25-hydroxyvitamin D. Am J Clin Nutr. 2012; 95:1055-105
14. Forman JP, Bischoff-Ferrari HA, Willett WC, Stampfer MJ, Curhan GC. Vitamin D intake and risk of incident hypertension: results from three large prospective cohort studies. Hypertension. 2005; 46:676-682.
15. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study. Arch Intern Med. 2008; 168:1174- 1180
16. Kane L, Moore K, Lütjohann D, Bikle D, Schwartz JB. Vitamin D3 effects on lipids differ in statin and non-statin-treated humans: superiority of free 25-OH D levels in detecting relationships. J Clin Endocrinol Metab. 2013 ;98(11):4400-9
17. Sathyapalan T, Shepherd J, Atkin SL, Kilpatrick ES. The effect of atorvastatin and simvastatin on vitamin D, oxidative stress and inflammatory marker concentrations in patients with type 2 diabetes: a crossover study. Diabetes Obes Metab. 2013;15(8):767-9
18. Rejnmark L, Vestergaard P, Heickendorff L, Mosekilde L. Simvastatin does not affect vitamin D status, but low vitamin D levels are associated with dyslipidemia: results from a randomized, controlled trial. Inter J Endocrin, 2010; 9: 1–6.

19. Choudhry NK, Levin R, Winkelmayer WC. Statins in elderly patients with acute coronary syndrome: an analysis of dose and class effects in typical practice. Heart. 2007;93(8):945-51
20. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P (2012) Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis, 22: 426-433
21. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81
22. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, Di Giovine G, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis. 2013;24(4):411-8.
23. De Luca G, Smit JJ, Ernst N, Suryapranata H, Ottervanger JP, Hoorntje JC, Dambrink JH, Gosslink AT, de Boer MJ, van 't Hof AW Impact of adjunctive tirofiban administration on myocardial perfusion and mortality in patients undergoing primary angioplasty for ST-segment elevation myocardial infarction. Thromb Haemost 2005; 93: 820-3.
24. De Luca G, Cassetti E, Verdoia M, Marino P. Bivalirudin as compared to unfractionated heparin among patients undergoing coronary angioplasty: A meta-analyis of randomised trials. Thromb Haemost. 2009;102(3):428-36
25. De Luca G, Navarese EP, Suryapranata H. A meta-analytic overview of thrombectomy during primary angioplasty. Int J Cardiol. 2013;166(3):606-12
26. Nichols M, Townsend N, Scarborough P, Rayner M. Cardiovascular disease in Europe 2014: epidemiological update. Eur Heart J 2014; 35:2950.
27. Lee JH, O'Keefe JH, Bell D, Hensrud DD, Holick MF. Vitamin D deficiency an important, common, and easily treatable cardiovascular risk factor? J Am Coll Cardiol. 2008 ;52(24):1949-56
28. Chitalia N, Ismail T, Tooth L, Boa F, Hampson G, Goldsmith D, Kaski JC, Banerjee D. Impact of vitamin D supplementation on arterial vasomotion, stiffness and endothelial biomarkers in chronic kidney disease patients. PLoS One. 2014 ;9(3):e91363
29. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, Galasso G, De Luca G. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014;44(7):634-42
30. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol. 2005;97:93-101
31. Silvagno F, De Vivo E, Attanasio A, Gallo V, Mazzucco G, Pescarmona G. Mitochondrial localization of vitamin D receptor in human platelets and differentiated megakaryocytes;. PLoS One. 2010 ;5:e8670
32. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, McKillop JH, Packard CJ; West of Scotland Coronary Prevention Study Group. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. 1995. Atheroscler Suppl. 2004;5(3):91-7
33. Lewis SJ, Moye LA, Sacks FM, Johnstone DE, Timmis G, Mitchell J, Limacher M, Kell S, Glasser SP, Grant J, Davis BR, Pfeffer MA, Braunwald E. Effect of pravastatin on cardiovascular events in older patients with myocardial infarction and cholesterol levels in the average range. Results of the Cholesterol and Recurrent Events (CARE) trial. Ann Intern Med. 1998;129(9):681-9
34. Hausenloy DJ, Yellon DM. Targeting residual cardiovascular risk: raising high-density lipoprotein cholesterol levels. Postgrad Med J. 2008 Nov;84(997):590-8
35. Liberopoulos EN, Makariou SE, Moutzouri E, Kostapanos MS, Challa A, Elisaf M. Effect of simvastatin/ezetimibe 10/10 mg versus simvastatin 40 mg on serum vitamin D levels. J Cardiovasc Pharmacol Ther. 2013 May;18(3):229-33
36. Ware WR. The JUPITER lipid lowering trial and vitamin D. Dermato-Endocrin, 2010; 2: 50–54
37. Ertugrul DT, Yavuz B, Cil H, Ata N, Akin KO, Kucukazman M, Yalcin AA, Dal K, Yavuz BB, Tutal E. STATIN-D study: comparison of the influences of rosuvastatin and fluvastatin treatment on the levels of 25 hydroxyvitamin D. Cardiovasc Ther. 2011;29(2):146-52
38. Staal A, Frith JC, French MH, et al. The ability of statins to inhibit bone resorption is directly related to their inhibitory effect on HMG-CoA reductase activity. J Bone Miner Res 2003;18:88–96
39. Thabit A, Alhifany A, Alsheikh R, Namnqani S, Al-Mohammadi A, Elmorsy S, Qari M, Ardawi M. Effect of simvastatin and atorvastatin on serum vitamin d and bone mineral density in hypercholesterolemic patients: a cross-sectional study. J Osteoporos. 2014;2014:468397
40. Anagnostis P, Adamidou F, Slavakis A, Polyzos SA, Selalmatzidou D, Panagiotou A, Athyros VG, Karagiannis A, Kita M. Comparative Effect of Atorvastatin and Rosuvastatin on 25-hydroxy-Vitamin D Levels in Non-diabetic Patients with Dyslipidaemia: A Prospective Randomized Open-label Pilot Study. Open Cardiovasc Med J. 2014;8:55-60
41. Schwartz JB. Effects of vitamin D supplementation in atorvastatin-treated patients: a new drug interaction with an unexpected consequence. Clin Pharmacol Ther, 2009; 85: 198–203
42. Xu Y, Hashizume T, Shuhart MC, et al. Intestinal and hepatic CYP3A4 catalyze hydroxylation of 1alpha,25-dihydroxyvitamin D(3): Implications for drug-induced osteomalacia. Mol Pharmacol 2006;69:56–65
43. Grimes DS. Are statins analogues of vitamin D. Lancet, 2006; 368: 83–86
44. Bhattacharyya S, Bhattacharyya K, Maitra A. Possible mechanisms of interaction between statins and vitamin D. QJM. 2012;105(5):487-91
45. Verdoia M, Pergolini P, Rolla R, Sartori C, Nardin M, Barbieri L, Schaffer A, Daffara V, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets 2016; Early Online 1-7, DOI:10.3109/09537104.2016.1149159
46. Siller-Matula JM, Gruber C, Francesconi M, Dechant C, Jilma B, Delle-Karth G, Grohs K, Podczeck-Schweighofer A, Christ G. The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary intervention. Clin Sci (Lond). 2015;128:121-30.

# Chapter 4:

## Vitamin D deficiency and periprocedural myocardial infarction in patients undergoing percutaneous coronary interventions

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### Abstract

Vitamin D deficiency has been implicated in the progression of atherosclerosis and acute thrombotic events. We aimed at evaluating the impact of vitamin D deficiency on periprocedural myocardial infarction (PMI) in patients undergoing percutaneous coronary interventions (PCI).

We included 934 patients undergoing non-urgent PCI. Assessment of myocardial biomarkers was performed from 6 to 48 hours after PCI. PMI was defined as Creatine Kinase-MB increase by 3 times the Upper Limit Normal or by 50% of an elevated baseline value, periprocedural myocardial damage as Troponin I increase by 3xULN or 50% of baseline.

Patients were divided according to Vitamin D tertiles values (<10.2ng/ml; 10.2-18.7ng/ml; ≥18.8ng/ml). Lower tertiles values of vitamin D were associated with age (p=0.04), female gender, (p=0.001), and a higher cardiovascular risk profile. Lower vitamin D levels related with PCI of descending anterior coronary artery or bypass vein grafts (p=0.03), treatment of bifurcations (p=0.05) and side branch loss (p=0.05) and inversely with direct stenting (p=0.002). However, lower Vitamin D levels did not influence the risk of PMI (adjusted OR [95%CI]=0.81[0.65,1.18],p=0.09) or periprocedural myocardial damage (adjusted OR[95%CI]=0.93[0.77,1.13],p=0.48). Similar results were achieved when considering the severity of vitamin D deficiency.

Therefore, in patients undergoing PCI, no association was observed between vitamin D deficiency and the risk of periprocedural MI and myocardial damage.

Keywords: vitamin D; periprocedural myocardial infarction; percutaneous coronary revascularization

## Background

Coronary artery disease is still the major determinant of mortality in developed countries, despite the great benefits in survival observed especially in the treatment of acute myocardial infarction [1,2]. Technological improvements and the introduction of innovative and more potent antithrombotic therapies have allowed an escalation in the complexity of percutaneous coronary interventions (PCI), therefore enhancing the risk of periprocedural complications [3].

In fact, periprocedural myocardial infarction (PMI) can affect up to 30% of procedures, translating into negative outcome effects [4,5]. Principal detectable causes include coronary dissections, distal embolization or spasm, although myocardial damage can occur even in apparently uneventful procedures, due to the disruption or thrombotic obstruction of coronary microcirculation [6]. Elevated platelet reactivity and inflammatory response have been claimed as major determinant of these events, with parameters as C-reactive protein having been demonstrated to predict the occurrence of myocardial injury post-PCI [7].

Great interests have been focused on Vitamin D (25OHD), for its multiple athero-protective and anti-inflammatory effects [8,9]. In fact, a long-term effect of vitamin D deficiency on the risk of cardiovascular disease is well established [10,11], while, few studies have so far addressed the role of vitamin D levels in patients undergoing PCI [12].

Aim of current study was to assess the impact of vitamin D levels on periprocedural myocardial infarction (PMI) in patients undergoing coronary percutaneous revascularization.

## Methods

Patients undergoing PCI at Ospedale “Maggiore della Carità” from January 2009 to January 2014 were prospectively included. Indication to PCI was both elective or acute coronary syndrome (UA/NSTEMI). NSTEMI patients were defined in case of chest pain at rest and cardiac biomarkers elevation >ULN

(0,04 µg/l for Troponin I and 5,00 µg/l for CK-MB, respectively), with or without electrocardiographic changes, and were undergoing elective coronary angiography after pharmacological stabilization. STEMI patients and hemodynamically unstable patients or those requiring urgent angioplasty soon after admission were excluded, as much as those patients with baseline raising values of cardiac enzymes, not allowing the interpretation of PCI related variations.

Diabetes mellitus was defined for previous diagnosis, use of specific hypoglycemic treatment (oral drug or insulin), fasting glycemia > 126 mg/dL in at least 2 repeated determinations or HbA1C > 6.5%. Hypertension was defined in case of systolic pressure > 140 mm Hg and/or a diastolic pressure >90 mmHg or a chronic treatment with antihypertensive medications [13]. We considered a diagnosis of chronic renal failure for a pre-PCI glomerular filtrate (GFR) < 60 ml/min/1.73m2 by the MDRD formula (Modification of Diet in renal Disease) [14]. Patients treated with vitamin D supplementation at admission or with unavailable vitamin D status was were excluded.

The study was approved by our local Ethical Committee, informed consent was obtained for the inclusion of the patients and the study was performed conform the declaration of Helsinki. According to guidelines, high-dose bolus of clopidogrel (600 mg) was administered to all patients at the time of hospitalization or before coronary angioplasty.

### Biochemical measurements

Main chemistry parameters, including vitamin D, were assessed from fasting blood samples at admission [15]. and were determined by standard methods. All blood samples were processed within 2 h from drawing. Cardiac biomarkers (Troponin I and CK MB) were dosed at baseline, before coronary revascularization, and at 6, 12, 24 and 48 h post PCI.

Vitamin D levels were measured by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). Normal laboratory range for 25OH D is from 30 to 100 ng/ml, vitamin D severe deficiency was considered for values < 10 ng/ml according to literature reference [16].

## Coronary angiography and PCI

Coronary angiography was performed preferentially through a trans-radial approach by the Judkins technique, with 6-French catheters. Quantitative coronary angiography for the correct sizing of the lesions was performed by an automatic edge-detection system (Siemens Acom Quantcor QCA, Erlangen, Germany) [17]. Coronary angioplasty was performed by experienced operators with standard techniques. Indication to stenting, use of drug-eluting stents and implantation techniques, or the use of special techniques as rotational atherectomy, intravascular imaging or glycoprotein IIb-IIIa inhibitors, was left at the decision of the operators.

## Study Endpoints

Our primary endpoint was the occurrence of periprocedural MI, defined as CK-MB mass elevation $\geq$ 3 times the upper limit normal (ULN) or an increase by 50% of a baseline elevated value, if stable or falling at baseline. Secondary endpoint was myocardial damage, considered for a periprocedural raise of troponin I $\geq$ 3 x ULN or by 50% of the pre-procedural value, if yet > 0.04 ng/ml.

## Statistical analysis

SPSS 17.0 and 22.0 statistical package were used for statistical analysis. Continuous data were reported as mean $\pm$ SD and categorical data in percentage. Analysis of variance and the chi-square test (or Fisher-test) were applied for continuous and categorical variables, respectively. Bonferroni correction was used for multiple comparison ANOVA. We conducted a multiple logistic regression analysis to evaluate the relationship between vitamin D levels and periprocedural myocardial necrosis or infarction after the inclusion in a “block” model of all the potential confounders (clinical and angiographic significant differences at univariate analysis). A propensity score was calculated by a forward conditional multivariable regression model and applied to perform a subgroup analysis assessing the impact of vitamin D on periprocedural MI according to the values of propensity score.

## Results

Our population consists of 1132 patients undergoing PCI. Among them 46 (4.7%) received chronic therapy with vitamin D and were excluded from data analysis, as much as the 152 STEMI patients and patients undergoing urgent PCI, with a final population of 934 patients. Mean vitamin D levels in our population were 16.1±9.3 ng/ml.

**Table 1.** Clinical characteristics according to Vitamin D tertiles.

| Baseline clinical characteristics | I tert (<10.2 ng/ml) | II tert (10.2-18.7 ng/ml) | III tert (≥18.8 ng/ml) | P value |
|---|---|---|---|---|
| Age (mean±SD) | 68.3±11.9 | 66.2±11.5 | 66.7±10.2 | 0.04 |
| Male Sex (%) | 69.2 | 84.6 | 70.8 | 0.001 |
| BMI (mean±SD) | 27±4.6 | 27.3±4.2 | 27.3±4 | 0.60 |
| Hypercholesterolemia(%) | 64.7 | 62.1 | 57 | 0.05 |
| Diabetes mellitus (%) | 41.5 | 38.5 | 37.8 | 0.34 |
| Renal failure (%) | 22.3 | 15.7 | 15.5 | 0.03 |
| Family history of CAD (%) | 30.5 | 35 | 30.9 | 0.92 |
| Smokers (%) | | | | 0.69 |
| Active smokers | 28.2 | 26.6 | 18.9 | |
| Previous smokers | 22.3 | 25.6 | 28.3 | |
| Arterial hypertension (%) | 74.7 | 74.5 | 73.4 | 0.71 |
| History of MI (%) | 30.2 | 25.1 | 24.2 | 0.09 |
| Previous PCI (%) | 32.8 | 36.9 | 35.4 | 0.50 |
| Previous CABG (%) | 16.6 | 12.7 | 12.4 | 0.14 |
| Autumn/winter admission | 58.8 | 50.2 | 46.7 | 0.01 |
| Indication to angiography | | | | <0.001 |
| Stable angina/ silent ischemia(%) | 20.9 | 28.2 | 34.3 | |
| ACS (%) | 70.6 | 65.3 | 62.6 | |
| Other (%) | 8.5 | 6.5 | 3.1 | |
| Therapy at admission | | | | |
| ACE inhibitors(%) | 40.7 | 40.5 | 34.4 | 0.1 |
| ARB (%) | 18.7 | 20.6 | 21.1 | 0.46 |
| Beta blockers (%) | 49.2 | 59.5 | 54.4 | 0.19 |
| Nitrates (%) | 33.1 | 38.6 | 37.2 | 0.30 |
| Statins (%) | 48.2 | 59.2 | 57.3 | 0.02 |
| Calcium antagonists (%) | 19.3 | 18 | 21.4 | 0.52 |
| Diuretics (%) | 32.1 | 24.5 | 18.3 | <0.001 |
| ASA (%) | 59.7 | 65.4 | 64.4 | 0.23 |
| Clopidogrel (%) | 21 | 23.9 | 25.7 | 0.17 |

| Main chemistry | | | | |
|---|---|---|---|---|
| Glycaemia (mg/dl) | 129±61.8 | 128.8±58.8 | 124.9±41.4 | 0.56 |
| HbA1c (mmol/l) | 46.7±15.6 | 46.2±15.3 | 43.6±10 | 0.01° |
| Creatinine (mg/dL) | 0.89±0.50 | 0.84±0.48 | 0.81±0.47 | 0.11 |
| Platelets (10^5/ml) | 221±80.6 | 214.4±56.4 | 211.5±66.2 | 0.16 |
| Haemoglobin (g/dl) | 12.7±1.8 | 13.1±1.7 | 13.2±1.6 | 0.002*,° |
| WBC (10^3/ml) | 7.8±2.8 | 7.3±2.5 | 7.3±2.5 | 0.01° |
| Total cholesterol (mg/dl) | 167.8±46.6 | 159±45.4 | 154.4±36.3 | <0.001*,° |
| LDL cholesterol (mg/dl) | 97.7±38.5 | 92.2±42.9 | 87.8±33.4 | 0.006° |
| Fibrinogen (mg/dl) | 425.9±137.9 | 401.5±136.7 | 391.3±127.1 | 0.006° |
| C reactive protein (mg/dl) | 1.38±0.63 | 1.07±0.54 | 1.07±0.46 | 0.02 |
| Baseline Troponin I (ng/ml) | 4.5±19.2 | 4.5±30.8 | 4.6±27.2 | 0.98 |
| Baseline CK-MB (µg/l) | 27.7±68.4 | 22±58.2 | 24.2±58.6 | 0.67 |

*CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; EF = Ejection Fraction; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers; ASA = Acetylsalicylic Acid; LDL = Low-Density Lipoproteins.*

** p<0.05 for I vs II tertile, †p<0.05 for II vs III tertile, ° p<0.05 for I vs III tertile*

Patients were divided according to vitamin D tertiles values (I tertile: <10.2 ng/ml; II tertile: 10.2-18.7 ng/ml; III tertile: ≥ 18.8 ng/ml). Severe hypovitaminosis D (<10 ng/ml) was found in 302 patients (32.3%), whereas 47.9% of patients displayed values < 20 ng/ml.

As expected, a seasonal variation in 25OHD was observed if the measurement of vitamin D was performed in autumn/winter (September to March) vs spring/summer (March to September) (mean value 14.9±8.9 vs 17.1±9.6 ng/ml, p<0.001, respectively), although largely remaining in the range of hypovitaminosis D (<30 ng/ml) for the majority of patients (93% vs 88.9%, p=0.03 for autumn/winter seasons vs spring/summer seasons, respectively).

Table 1 displays main clinical and demographic features. Hypovitaminosis D was associated with age (p=0.04), female gender, (p=0.001), hypercholesterolemia (p=0.05), renal failure (p=0.03), acute coronary syndrome at presentation (p<0.001), treatment with diuretics (p<0.001), higher levels of white blood cells (p=0.01), HbA1c (p=0.01), fibrinogen (p=0.006), C- reactive protein (p=0.02), cholesterol (p<0.001) and lower haemoglobin values (p=0.002) and statins use (p=0.02).

Table 2. Angioplasty Features according Vitamin D tertiles (per lesion).

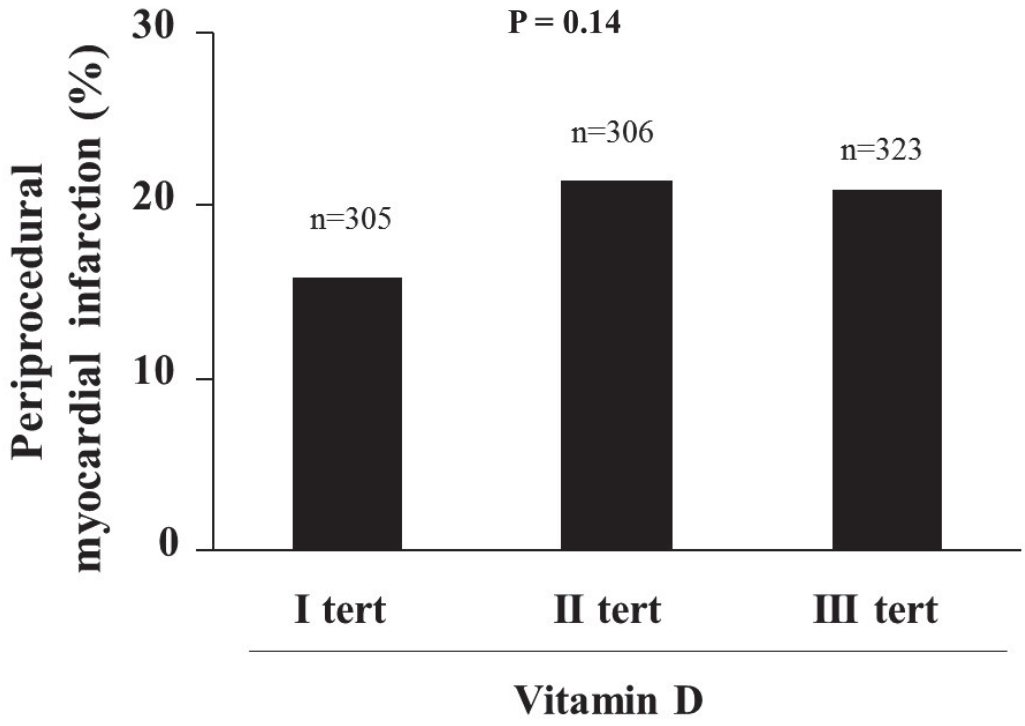
| Procedural features | I tert (<10.2) N= 394 | II tert (10.2-18.7) N= 396 | III tert (≥18.8) N= 394 | P value |
|---|---|---|---|---|
| Severe CAD (% ) § | 33.9 | 31.1 | 33.2 | 0.99 |
| Multivessel Disease (%) § | 61.8 | 59.5 | 54.8 | 0.32 |
| Gp IIb-IIIa inhibitors (%)§ | 49.4 | 51.9 | 46.4 | 0.52 |
| Clopidogrel bolus> 6 h | 17.9 | 18.5 | 15.5 | 0.95 |
| Multivessel PCI (%)§ | 27.9 | 29.6 | 26.5 | 0.72 |
| Lesion length (mm ± SD) | 23.3±14 | 23.8±14.2 | 23.4±15.2 | 0.90 |
| Target Vessel diameter | 2.7±0.7 | 2.7±0.7 | 2.7±0.6 | 0.57 |
| % stenosis (± SD) | 88±9.9 | 89.5±9 | 88.9±9.7 | 0.22 |
| Target vessel | | | | 0.03 |
| Right Coronary Artery (%) | 18.8 | 15.9 | 16.2 | |
| Left Main (%) | 2.8 | 4.3 | 2 | |
| Left Anterior Descending | 33 | 30.6 | 28.4 | |
| Circumflex branch (%) | 16.5 | 17.9 | 15 | |
| Saphenous venous graft | 3.8 | 3.3 | 2.5 | |
| Antero-lateral branch (%) | 12.7 | 16.7 | 12.9 | |
| Eccentric plaque (%) | 99.1 | 98 | 97.4 | 0.08 |
| Type C lesions (%) | 32.8 | 34.8 | 34.4 | 0.64 |
| Calcifications (% ) | 9.6 | 10 | 14.1 | 0.07 |
| Thrombus (% ) | 9.9 | 8.8 | 7.5 | 0.30 |
| TIMI flow pre-PCI <3 (% ) | 18.2 | 16.5 | 18.2 | 0.75 |
| Instent restenosis (% ) | 5.5 | 6.9 | 8.5 | 0.13 |
| Chronical occlusion (% ) | 7 | 6.3 | 8.6 | 0.45 |
| Bifurcations (% ) | 29.9 | 30.9 | 22.7 | 0.05 |
| Predilatation(% ) | 65.3 | 62.3 | 63.3 | 0.59 |
| Direct stenting ( %) | 28.7 | 32.6 | 27.7 | 0.002 |
| BMS (%) | 27.5 | 21.4 | 20.9 | 0.06 |
| Max inflation (atm± SD) | 21.4±3.6 | 21.9±3.8 | 21.6±3.4 | 0.25 |
| Kissing balloon (% ) | 16.7 | 23 | 22.1 | 0.11 |
| Thrombectomy ( %) | 4.3 | 3 | 2.5 | 0.24 |
| Rotablator (% ) | 1 | 0.7 | 1 | 0.8 |
| Drug-Eluting Stent (% ) | 74.2 | 79 | 80.2 | 0.11 |
| TIMI post PCI <3 (%) | 16.8 | 16.2 | 21.1 | 0.06 |
| Any dissection (% ) | 3.3 | 2.6 | 2.3 | 0.31 |
| Coronary perforation (% ) | 2.1 | 0.6 | 1.7 | 0.63 |
| Distal embolization (% ) | 2.4 | 0.9 | 0.7 | 0.06 |
| Additional stent required | 2.2 | 2.1 | 1.3 | 0.50 |
| Side branch loss (% ) | 1.2 | 0.6 | 0 | 0.05 |

§ per patient definition

Table 2 lists main angiographic and procedural features (per lesion). Lower vitamin D levels related with PCI of lesions on descending anterior coronary artery or bypass conduct (p=0.03), treatment of bifurcations (p=0.05) and side branch loss (p=0.05) and inversely with direct stenting (p=0.002). No difference was found according to adjunctive pharmacological therapy during PCI.

As displayed in Figure 1, Vitamin D levels did not influence the risk of PMI (15.7% vs 21.4% vs 20.9%, p=0.14, OR[95%CI]= 0.88 [0.64,1.17], p=0.08). No impact was found for periprocedural myocardial damage (62.8% vs 68% vs 66.4%, P=0.38, OR[95%CI]=0.90 [0.76,1.07], p=0.22) (Figure 2).

**Figure 1.** Bar graphs show the prevalence (with standard error) of periprocedural myocardial infarction (defined as Creatine Kinase-MB by 3 times the Upper Limit Normal or by 50% of baseline value), according to vitamin D levels (tertiles).




As shown in Table 3, the results did not change after correction for baseline differences. In fact, no difference in the levels of vitamin D was identified in patients experiencing or not PMI (16±9 vs 15.4±8.9 ng/ml, p=0.42)

Figure 2. Bar graphs show the prevalence (with standard error) of periprocedural myocardial damage (defined as Troponin I increase by 3 timed the Upper Limit Normal or by 50% of baseline value) according according to vitamin D levels (tertiles).

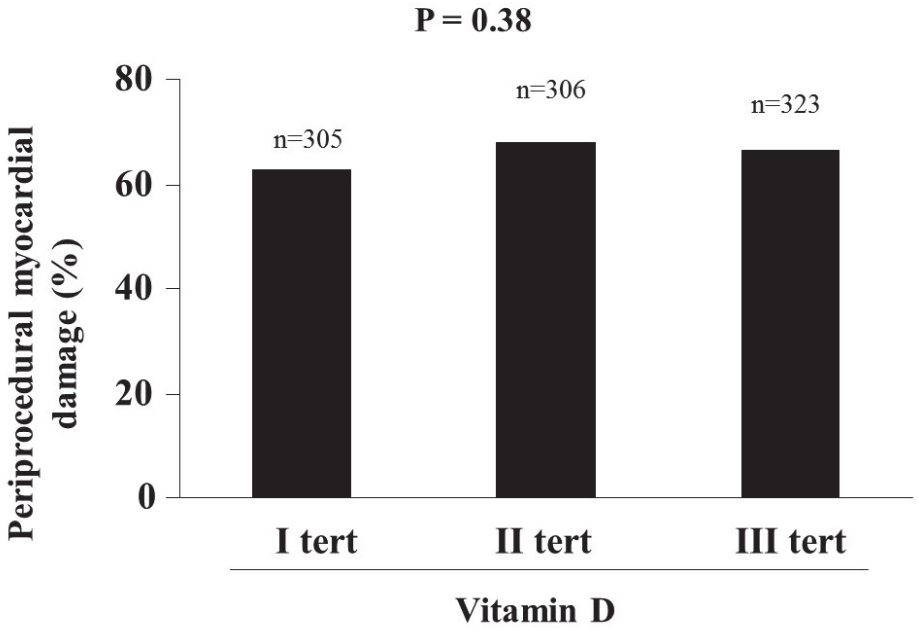
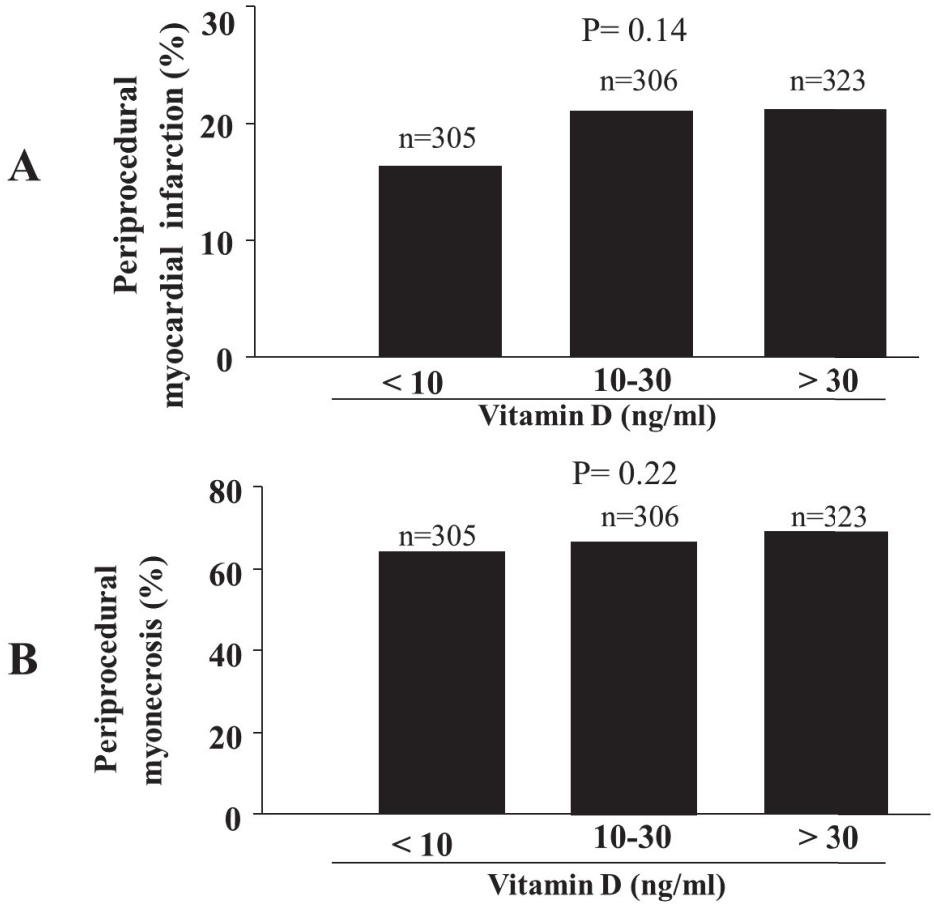



Figure 3. Bar graphs show the prevalence (with standard error) of periprocedural myocardial infarction (A, upper graph) and myonecrosis (B, lower graph) according to vitamin D deficiency severity.



Similar results were obtained in the 317 patients with stable coronary artery disease (PMI:12.3% vs 17.9% vs 13.5%, p=0.85; Myocardial damage: 71.3% vs 72.9% vs 71.7%, p=0.96) and among the 617 ACS patients (PMI: 17.6% vs 24.6% vs 22.8%, p=0.16; Myocardial damage: 55.5% vs 62.4% vs 61.5%, p=0.27), that were confirmed after correction for baseline differences (Table 3).

Results did not change when applying the cut-offs for the definition of the severity of vitamin D deficiency (<10 ng/ml- 10-30 ng/ml, > 30 ng/ml) (15.9% vs 21.1% vs 21%, p=0.14; for PMI and 62.8% vs 66.8% vs 69%, p=0.22, for myocardial damage; Figure 3) and at multivariate analysis (Table 3). Results were comparable also if considering patients with severe deficiency (<10 ng/ml) vs the rest of the population, as shown in Table 3.

Table 3. Multivariate analysis results for the risk of periprocedural myocardial infarction and myonecrosis

| Variable | Adjusted* OR | CI | P value | P interaction |
|---|---|---|---|---|
| **Periprocedural myocardial infarction** | | | | |
| *Vitamin D (across tertiles)* | 0.81 | [0.65,1.18] | 0.09 | |
| *Vitamin D deficiency (severity)* | 0.69 | [0.45;1.08] | 0.11 | |
| *Vitamin D deficiency (< vs ≥ 10 ng/ml)* | 0.69 | [0.847, 1.03] | 0.07 | |
| *Stable coronary artery disease* | 0.96 | [0.66, 1.41] | 0.86 | 0.17 |
| *Acute coronary syndromes* | 0.77 | [0.59, 1.05] | 0.08 | |
| **Periprocedural myocardial damage** | | | | |
| *Vitamin D (across tertiles)* | 0.93 | [0.77,1.13] | 0.48 | |
| *Vitamin D deficiency (severity)* | 0.87 | [0.61-1.25] | 0.47 | |
| *Vitamin D deficiency (< vs ≥ 10 ng/ml)* | 0.84 | [0.63, 1.13] | 0.25 | |
| *Stable coronary artery disease* | 0.92 | [0.69, 1.23] | 0.58 | 0.16 |
| *Acute coronary syndromes* | 0.93 | [0.71,1.21] | 0.23 | |

** after correction for: age[1], gender, hypercholesterolemia, renal failure, acute coronary syndrome, statins, diuretics, white blood cells[1], HbA1c[1], fibrinogen, C- reactive protein[1], cholesterol, haemoglobin values and season of vitamin D dosing, treated segment, bifurcation lesion, side branch loss and direct stenting.*

In addition, similar results were achieved when comparing patients according to patients' risk profile. A propensity score was calculated by multivariate regression analysis based on independent predictors of periprocedural myocardial infarction (previous MI: OR[95%%CI]= 0.46[0.27-0.77],

p=0.003; therapy with angiotensin-receptor blockers: OR[95% CI]= 0.47[0.27-0.81], p=0.007; thrombus: OR[95%%CI]= 1.81[1.03-3.17], p=0.04; kissing balloon: OR[95%%CI]= 1.63[1.04-2.54], p=0.03; slow- flow: OR[95%%CI]= 6.2[2.6-14.73], p<0.001) . In fact, a similar rate of PMI was observed according to median values of propensity score (< or ≥0), for patients with severe vitamin D deficiency vs the rest of the population (low propensity score: 6.7% vs 11.7%, p=0.21; high propensity score: 19.5% vs 24.4%, p=0.21, p interaction =0.09), Results did not change also for periprocedural myocardial damage (low propensity score: 64.8% vs 64.5%, p=0.17; high propensity score: 61.1% vs 67.2%, p=0.99, p interaction = 0.28).

Moreover, no difference was observed in the subgroup of patients treated with GPIIbIIIa inhibitors (n=422) in terms of periprocedural MI (16.4% vs 18.9% vs 21.7%, p=0.11) and troponin-myocardial damage (66.2% vs 66.9% vs 73.4%, p=0.38) and among patients not receiving periprocedural GPIIbIIIa inhibitors (13.2% vs 21.3% vs 13.2%, p=0.14 for PMI and 59.7% vs 66.7% vs 63.5%, p=0.49 for myocardial damage).

## Discussion

Present study firstly addresses the impact of vitamin D levels on the occurrence periprocedural myocardial infarction. Our principal finding is that lower levels of 25OH D do not influence the risk of myocardial damage in patients undergoing PCI.

Percutaneous coronary interventions (PCI) are nowadays the main revascularization strategy among patients with coronary artery disease. However, pharmacological and technical innovations [18,19] have not completely overcome the limitations of PCI in more complex patients, where still suboptimal results can be observed [20,21].

In particular, procedural complications, as distal embolization, flow-limiting dissections or side branch loss are well established causes for the occurrence of periprocedural myocardial infarction (PMI), a

feared event still occurring in several patients undergoing PCI and with an unfavorable impact on prognosis [22].

However, a silent myocardial damage has been detected even apparently uncomplicated PCI, probably consequence of a coronary microvascular thrombosis [23], that can certainly be favored by elevated platelet reactivity and enhanced inflammatory status. However, very few data have been reported on the role biomarkers possibly predicting such complication [24].

Great attention has been recently addressed to vitamin D, for its potential effects on cardiovascular disease [25] in addition to the endemic dimension of the problem of hypovitaminosis D, [26, 27]. In fact, previous studies reported serum levels of vitamin D being inversely associated with the extent of coronary disease [28] and with indirect markers of atherosclerosis, as coronary calcifications or carotid IMT [29]. Moreover, in the large Multi-Ethnic Study of Atherosclerosis (MESA), lower serum 25OH D concentration increased the risk of cardiovascular events at 8 years follow-up among white race participants, where mean value of vitamin D was 10.6 ng/ml [30]. More recently, Naesgaard reported Vitamin D levels to predict mortality in females admitted for acute coronary syndrome, in a population where about 50% of the women displayed vitamin D deficiency [31]. Indeed, lower vitamin D levels have been linked to the pathogenesis of established cardiovascular risk factors, as hypertension [32] or hypercholesterolemia [33] and to the etiology of atherothrombosis, enhancing endothelial dysfunction and the inflammatory response [34,35], while contrasting data have been obtained for the long-term risk of diabetes [36]. In a previous study, Gupta et al. [37] reported that inadequate vitamin D levels could potentiate the risk of coronary restenosis after stent implantation in a swine model, by modulating neointimal and smooth muscle cells proliferation through atherogenic cytokines, including TNF-a. Similar results were suggested by Monraats et al in a model evaluating the genetic predictors of instent restenosis [12]. Moreover, vitamin D has been reported

to inhibit the reactivity of platelets both in vitro and in vivo [34]. However, the role of vitamin D levels on periprocedural myocardial damage has never been assessed.

We currently confirmed the significant prevalence of hypovitaminosis D among the white population, and especially among patients with CAD, as those included in our study. Moreover, we found a significant inverse relationship among 25OH D levels and ageing, female gender and renal function. Lower vitamin D levels were associated to significantly higher cholesterol and LDL levels, despite statins treatment, thus further pointing at a potential interaction of vitamin D with lipids metabolism, as previously suggested [38].

Finally, we confirmed a significant association of hypovitaminosis D with acute coronary syndromes and with the elevation of inflammatory biomarkers [37].

However, vitamin D levels did not impact on periprocedural MI or myocardial damage, even after correction for baseline differences, and even when considering the definition of the severity of vitamin D deficiency. In addition, similar results were obtained in patients with either acute or elective presentation.

Several explanations can be provided for our current negative result. Indeed, PMI recognizes a different pathogenesis from atherosclerosis and restenosis and probably coronary anatomy and lesion complexity had in our patients a more relevant impact on PMI. Adjunctive pharmacological therapies may have avoided a large quote of events in our patients, and in particular for statins, a category of drugs that are known to prevent myocardial damage during PCI and whose pleiotropic effects have been linked to a rise in the levels of vitamin D [38].

In addition, the extensive use of GPIIbIIIa inhibitors in almost 50% of PCI may have helped to prevent the thrombotic phenomena, that are held responsible for PMI. Finally, recent evidence has emerged on a paracrine, local-tissue production of the active vitamin D form, that could have offered a myocardial protection from periprocedural injury, independently from circulating levels of 25OHD,

although this intriguing hypothesis still needs to be verified in large studies [39]. Nevertheless, despite the association of higher levels of vitamin D with a lower cardiovascular risk-profile, according to the present study, vitamin D supplementation should not be overlooked as a potential strategy for the prevention of periprocedural myocardial infarction among patients undergoing PCI.

## Study limitations

A major limitation to our study is certainly represented by the absence of a long-term follow-up. Therefore we cannot excluded any potential prognostic effect of low vitamin D on the occurrence of thrombotic complications at distance. In addition, the long period of patients recruitment, with seasonal variability of vitamin D levels could have slightly modified our results, although inadequate vitamin D levels were observed in the vast majority of our patients. On the other side, the high prevalence of hypovitaminosis D could have conditioned our negative observations. However, previous large studies (NHANES III, Framingham Offspring Study; [40-41]) have suggested that the increase in cardiovascular risk could be observed even for very low vitamin D levels (below 15 or 20 ng/ml), that were reached in about 30% of patients in the study of Wang et al. and half of our study population. Therefore, we expect that we would have been able to detect any impact of vitamin D on PMI, if present. Nevertheless, the exact levels of 25OH D needed for cardiovascular protection are still matter of debate, and therefore we cannot exclude that a vitamin D supplementation in our patients undergoing PCI would have prevented a quote of periprocedural complications. Moreover, life-style and genetic factors could have influenced the levels and effectiveness of vitamin D. In fact, several polymorphic variants of vitamin D binding protein and vitamin D receptor can regulate the hormonal effects of 1,25OH2D and the pathways leading to activation and deactivation of vitamin D into its active hydroxylized metabolites. In fact, we excluded patients receiving chronic supplementation with vitamin D, but we did not collect data on sporadic treatments with multivitamins, that could have indeed influenced the levels of vitamin D in certain patients.

The inclusion of patients with previous cardiovascular events, and especially those with a previous myocardial infarction or complex coronary anatomy, such as coronary artery bypass grafts, may have represented a population at higher risk of periprocedural myocardial injury, although these factors were equally distributed across vitamin D tertiles in our population. Moreover, patients with ST-segment elevation myocardial infarction or persistent ischemia, requiring urgent revascularization, were not included and therefore we did not expect that time from symptoms onset to balloon inflation might have played a relevant role our study. In addition, we could not define the cause of PMI in the whole cohort of our patients, since evident periprocedural complications occurred only in a minor proportion of our patients, although not differing according to the levels of vitamin D.

Furthermore, we could not apply the most recent definition of periprocedural myocardial infarction to our study, requiring the additional evaluation of symptoms, ECG and echocardiogram in addition to cardiac biomarkers, since these parameters were not required in the universal definition of PMI [42] that was in use at the beginning of the study and were therefore not available in the entire cohort of patients. Moreover, despite the 2007 Universal Definition defines Type 4a MI as either CK-MB or troponin elevation of >3× upper reference limit (URL), we preferred to use the CK-MB, over troponin. as a biomarker of PMI, being associated with the outcome for our primary study endpoint, as validated in principal studies on the topic. However, the “troponin” periprocerdural myocardial damage was also assessed, as a secondary study endpoint (5).

## Conclusions.

In our study vitamin D deficiency was frequent among patients undergoing non-urgent PCI. However, we observed no association between vitamin D levels and the risk of periprocedural MI and myocardial damage.

# References


1. De Luca G, Suryapranata H, Marino P Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status.. Prog Cardiovasc Dis 2008; 50: 352-382.
2. Di Lorenzo E, De Luca G, Sauro R, Varricchio A, Capasso M, Lanzillo T, Manganelli F, Mariello C, Siano F, Pagliuca MR, Stanco G, Rosato G. The PASEO (PaclitAxel or Sirolimus-Eluting Stent Versus Bare Metal Stent in Primary Angioplasty) Randomized Trial. JACC Cardiovasc Interv 2009; 2: 515-523.
3. Capozzolo C, Piscione F, De Luca G, Cioppa A, Mazzarotto P, Leosco D, Golino P, Indolfi C, Chiariello M. Direct coronary stenting: effect on coronary blood flow, immediate and late clinical results. Catheter Cardiovasc Interv 2001; 53: 464-473
4. Feldman DN, Kim L, Rene AG, Minutello RM, Bergman G, Wong SC. Prognostic value of cardiac troponin-I or troponin-T elevation following nonemergent percutaneous coronary intervention: a meta-analysis. Catheter Cardiovasc Interv. 2011; 77: 1020-1030
5. Califf RM, Abdelmeguid AE, Kuntz RE, Popma J, Davidson CJ, Koen EA, Kleiman NS, Mahaffey KW, Topol EJ, Pepine CJ, Lipicky RJ, Granger CB, Harrington RA, Tardiff BE, Crenshaw BS, Barman RP, Zuckerman BD, Chaitman BR, Bitti JA, Magnus Ohman E Myonecrosis after revascularization procedures. J Am Coll Cardiol 1998; 31:241-251
6. Michaels AD, Gibson CM, Barron HV. Microvascular dysfunction in acute myocardial infarction: focus on the roles of platelet and inflammatory mediators in the no-reflow phenomenon. Am J Cardiol. 2000; 85:50b–60b
7. Niccoli G, Sgueglia GA, Latib A, Crea F, Colombo A; CACTUS Study Group. Association of baseline C-reactive protein levels with periprocedural myocardial injury in patients undergoing percutaneous bifurcation intervention: a CACTUS study subanalysis. Catheter Cardiovasc Interv. 2014;83(1):E37-44
8. Buitrago CG, Arango NS, Boland RL. 1α,25(OH)2D-dependent modulation of Akt in proliferating and differentiating C2C12 skeletal muscle cells. J Cell Biochem. 2012;113:1170-1181
9. van Etten E, Mathieu C. Immunoregulation by 1,25-dihydroxyvitamin D3: basic concepts. J Steroid Biochem Mol Biol. 2005;97:93-101
10. Giovannucci E, Liu Y, Hollis BW, Rimm EB. 25-hydroxyvitamin D and risk of myocardial infarction in men: a prospective study. Arch Intern Med. 2008; 168:1174- 1180
11. Ginde AA, Scragg R, Schwartz RS, Camargo CA Jr. Prospective study of serum 25-hydroxyvitamin D level, cardiovascular disease mortality, and all-cause mortality in older U.S. adults. J Am Geriatr Soc. 2009;57:1595-603.
12. Monraats PS, Fang Y, Pons D, Pires NM, Pols HA, Zwinderman AH, de Maat MP, Doevendans PA, DeWinter RJ, Tio RA, Waltenberger J, Frants RR, Quax PH, van der Laarse A, van der Wall EE, Uitterlinden AG, Jukema JW. Vitamin D receptor: a new risk marker for clinical restenosis after percutaneous coronary intervention. Expert Opin Ther Targets. 2010;14(3):243-251.
13. Perk J, De Backer G, Gohlke H, Graham I, Reiner Z, Verschuren WM, Albus C, Benlian P, Boysen G, Cifkova R, Deaton C, Ebrahim S, Fisher M, Germano G, Hobbs R, Hoes A, Karadeniz S, Mezzani A, Prescott E, Ryden L, Scherer M, Syvänne M, Scholte Op Reimer WJ, Vrints C, Wood D, Zamorano JL, Zannad F; Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice; European Association for Cardiovascular Prevention and Rehabilitation. European Guidelines on cardiovascular disease prevention in clinical practice (version 2012): The Fifth Joint Task Force of the European Society of Cardiology and Other Societies on Cardiovascular Disease Prevention in Clinical Practice (constituted by representatives of nine societies and by invited experts). Atherosclerosis. 2012;223(1):1-68
14. Levey AS, Coresh J, Greene T, Stevens LA, Zhang YL, Hendriksen S, Kusek JW, Van Lente F; Chronic Kidney Disease Epidemiology Collaboration. Using standardized serum creatinine values in the modification of diet in renal disease study equation for estimating glomerular filtration rate. Ann Intern Med. 2006;145(4):247-254. Erratum in: Ann Intern Med. 2008 Oct 7;149(7):519
15. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P; Novara Atherosclerosis Study Group (NAS). Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012;22(5):426-433
16. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357:266–281
17. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, Coppo L, Iorio S, Venegoni L, Rondano E, Dell'Era G, Rizzo C, Pergolini P, Monaco F, Bellomo G, Marino P Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis 2009; 206: 292-297
18. De Luca G, Bellandi F, Huber K, Noc M, Petronio AS, Arntz HR, Maioli M, Gabriel HM, Zorman S, DE Carlo M, Rakowski T, Gyongyosi M, Dudek D. Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab long-term results (EGYPT-ALT) cooperation: individual patient's data meta-analysis. J Thromb Haemost 2011; 9: 2361-2370
19. De Luca G, Dirksen MT, Spaulding C, Kelbaek H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Díaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Cortese G, Suryapranata H, Stone GW; Drug-Eluting Stent in Primary Angioplasty (DESERT) Cooperation. Drug-eluting vs bare-metal stents in primary angioplasty: a pooled patient-level meta-analysis of randomized trials. Arch Intern Med. 2012 Apr 23;172(8):611-621
20. De Luca G, Gibson CM, Bellandi F, Noc M, Dudek D, Zeymer U, Arntz HR, Cutlip D, Maioli M, Zorman S, Mesquita Gabriel H, Emre A, Rakowski T, Gyongyosi M, Huber K, Van't Hof AW. Diabetes mellitus is associated with distal embolization, impaired myocardial perfusion, and higher mortality in patients with ST-segment elevation myocardial infarction treated with primary angioplasty and glycoprotein IIb-IIIa inhibitors. Atherosclerosis 2009; 207: 181-185.
21. De Luca G, van 't Hof AW, Ottervanger JP, Hoorntje JC, Gosselink AT, Dambrink JH, de Boer MJ, Suryapranata H. Ageing, impaired myocardial perfusion, and mortality in patients with ST-segment elevation myocardial infarction treated by primary angioplasty. Eur Heart J. 2005 Apr;26(7):662-666
22. Hanna EB, Hennebry TA (2010) Periprocedural myocardial infarction: review and classification. Clin Cardiol.;33(8):476-483.
23. Muschart X, Slimani A, Jamart J, Chenu P, Dangoisse V, Gabriel L, Guédès A, Marchandise B, Schröder E. The different mechanisms of periprocedural myocardial infarction and their impact on in-hospital outcome. J Invasive Cardiol. 2012;24(12):655-660

24. Verdoia M, Schaffer A, Cassetti E, Barbieri L, Di Giovine G, Marino P, De Luca G; Novara Atherosclerosis Study Group (NAS). MTHFR polymorphism and risk of periprocedural myocardial infarction after coronary stenting. Nutr Metab Cardiovasc Dis. 2014 May;24(5):532-537
25. Nagpal S, Na S, Rathnachalam R (2005) Noncalcemic actions of vitamin D receptor ligands. Endocr Rev 26: 662–687
26. Holick, M. F., Matsuoka, L. Y., Wortsman, J. (1989). Age, vitamin D, and solar ultraviolet. The Lancet, 2, 1104–1105
27. Holick, M. F., MacLaughlin, J. A., Doppelt, S. H. Regulation of cutaneous previtamin D3 photosynthesis in man: skin pigment is not an essential regulator. Science, 211, 590–593.
28. Goleniewska BM, Kacprzak M, Zielinska M. Vitamin D level and extent of coronary stenotic lesions in patients with first acute myocardial infarction. Cardiol J 2014; 21(1):18–23
29. Watson KE, Abrolat ML, Malone LL, Hoeg JM, Doherty T, Detrano R, Demer LL. Active serum vitamin D levels are inversely correlated with coronary calcification. Circulation.1997 16; 96:1755-1760
30. Robinson-Cohen C, Hoofnagle AN, Ix JH, Sachs MC, Tracy RP, Siscovick DS, Kestenbaum BR, de Boer IH. Racial differences in the association of serum 25-hydroxyvitamin D concentration with coronary heart disease events. JAMA 2013; 310: 179-188
31. Naesgaard PA, León de la Fuente RA, Nilsen ST, Woie L, Aarsland T, Staines H, Nilsen DW. Vitamin d predicts all-cause and cardiac mortality in females with suspected acute coronary syndrome: a comparison with brain natriuretic Peptide and high-sensitivity C-reactive protein. Cardiol Res Pract. 2013;2013:398034
32. Forman JP, Bischoff-Ferrari HA, Willett WC, Stampfer MJ, Curhan GC. Vitamin D intake and risk of incident hypertension: results from three large prospective cohort studies. Hypertension. 2005; 46:676-682
33. Al-Daghri NM, Alkharfy KM, Al-Saleh Y, Al-Attas OS, Alokail MS, Al-Othman A, Moharram O, El-Kholie E, Sabico S, Kumar S, Chrousos GP. Modest reversal of metabolic syndrome manifestations with vitamin D status correction: a 12-month prospective study. Metabolism. 2012;61(5):661-666
34. *A*ihara K, Azuma H, Akaike M, Ikeda Y, Yamashita M, Sudo T, Hayashi H, Yamada Y, Endoh F, Fujimura M, Yoshida T, Yamaguchi H, Hashizume S, Kato M, Yoshimura K, Yamamoto Y, Kato S, Matsumoto T. Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice. J Biol Chem. 2004; 279:35798-35802
35. Tarcin O, Yavuz DG, Ozben B, Telli A, Ogunc AV, Yuksel M, Toprak A, Yazici D, Sancak S, Deyneli O, Akalin S. Effect of vitamin D deficiency and replacement on endothelial function in asymptomatic subjects. *J Clin Endocrinol Metab* 2009;94:4023–4030.
36. Danziger J, Biggs ML, Niemi M, Ix JH, Kizer JR, Djoussé L, de Boer IH, Siscovick DS, Kestenbaum B, Mukamal KJ. Circulating 25-hydroxyvitamin D is associated with insulin resistance cross-sectionally but not longitudinally in older adults: The Cardiovascular Health Study. Metabolism. 2013;62(12):1788-1794
37. Gupta GK, Agrawal T, Del Core MG, Hunter WJ 3rd, Agrawal DK. Decreased expression of vitamin D receptors in neointimal lesions following coronary artery angioplasty in atherosclerotic swine. PLoS One. 2012;7(8):e42789.
38. Kane L, Moore K, Lütjohann D, Bikle D, Schwartz JB. Vitamin D3 effects on lipids differ in statin and non-statin-treated humans: superiority of free 25OH D levels in detecting relationships. J Clin Endocrinol Metab. 2013;98(11):4400-4409
39. Mahdavi K, Amirajam Z, Yazdankhah S, Majidi S, Adel MH, Omidvar B, Alasti M. The prevalence and prognostic role of vitamin D deficiency in patients with acute coronary syndrome: a single centre study in South-West of Iran. Heart Lung Circ. 2013;22(5):346-351;
40. Martins D, Wolf M, Pan D, et al. Prevalence of cardiovascular risk factors and the serum levels of 25-hydroxyvitamin D in the United States: data from the Third National Health and Nutrition Examination Survey. Arch Intern Med. 2007;167(11):1159-1165
41. Wang TJ, Pencina MJ, Booth SL, et al. Vitamin D deficiency and risk of cardiovascular disease. Circulation. 2008;117(4):503–511
42. Thygesen K, Alpert JS, White HD; Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction.. Universal definition of myocardial infarction. Eur Heart J. 2007;28(20):2525-2538

Part 3

# *Genetic modulation of vitamin D levels*

# Chapter 5:

# Impact of polymorphism rs7041 and rs4588 of Vitamin D Binding Protein on the extent of coronary artery disease

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## ABSTRACT

**Background.** 25-hydroxyvitamin D deficiency represents a widespread social problem but also an emerging risk factor for cardiovascular disease. Genetic variants of the Vitamin D Binding Protein (VDBP), the main transporter of vitamin D in the bloodstream, have been shown to account for a significant variability in the levels and systemic effects of vitamin D. We investigated whether the single nucleotide polymorphisms, rs7041 and rs4588, of VDBP are associated to the prevalence and extent of coronary artery disease.

**Methods** A consecutive cohort of patients undergoing coronary angiography in a single centre were included. Significant CAD was defined as at least 1 stenosis > 50%, severe CAD for as left main and/or three-vessel disease. VDBP genetic status was assessed by polymerase chain reaction and restriction fragment length polymorphism technique.

**Results** We included 1080 patients, 57% carried the mutated G allele of rs7041, whereas 22% carried the A allele of rs4588. Higher levels of C- reactive protein were observed in the carriers of G allele of rs7041 (p=0.02), whereas 25-hydroxyvitamin D levels were similar across groups. Higher prevalence of lesions in the left anterior descending artery and a longer lesion length were observed in "A" carriers for rs4588 (p=0.04 e p=0.03, respectively). On the contrary, a higher prevalence of bifurcation lesions and chronic occlusions was observed in G carriers (p=0.002 and p=0.01

respectively). Both polymorphisms of VDBP did not affect the prevalence of CAD (rs7041: 79.1% TT vs 80.3% TG vs 78.5% GG, p=0.81; rs4588= 80.3% CC vs 78.5% AC+AA, p=0.49) and severe CAD, (rs7041: 31.1% TT % vs 31.3% TG vs 30.6% GG, p=0.88; rs4588: 32.2% CC vs 29.3% AC+AA, p=0.31). Results were confirmed at multivariate analysis, for both rs7041 and rs4588. However, when including the levels of 25-hydroxyvitamin D to the multivariate model, we observed that 25(OH)D status and not genetic variants of VDBP were significantly associated to CAD (25-hydroxyvitamin D OR [95% CI] = 0.99 [0.97-1.0], p=0.05; rs7041 TG: OR [95% CI] = 1.26 [0.73-2.19], p=0.41; rs7041 GG: OR [95% CI] = 1.25 [0.82-1.91], p=0.30; rs4588 AC+AA: OR [95% CI] = 0.76 [0.51-1.13], p=0.18).

Conclusion This study showed in a large cohort of patients undergoing coronary angiography, that the polymorphisms rs7041 and rs4588 of VDBP are not associated with the levels of 25-hydroxyvitamin D nor with the prevalence and extent of CAD. In fact, 25-hydroxyvitamin D levels but not VDBP genetic status independently predicted the occurrence of coronary lesions at angiography.

Keywords: Atherosclerosis; Vitamin D; VDBP; Polymorphisms

## INTRODUCTION

Recent advances in the fields of pharmacological treatment and percutaneous coronary revascularization have reduced overall mortality but not the burden of acute cardiovascular events, in patients with coronary artery disease (CAD) [1,2,3], and especially among higher-risk subsets of patients [4,5,6]. Therefore, in the last years, increasing efforts have been accomplished to identify correlates of coronary artery disease beyond well-known risk factors. Several markers of atherosclerosis have been proposed, although allowing only an early identification of cardiovascular disease, rather than its real prevention [7]. Thereby, a special attention has been paid to genetics, and especially to those common variants, the single nucleotide polymorphisms (SNP), potentially involved in the pathogenesis of atherosclerosis.

Vitamin D is a group of fat-soluble molecules and plays role of steroid hormone, regulating calcium and phosphorus metabolism, but also playing a key function in cardiovascular health, improving endothelial function, decreasing inflammatory response with a consequential anti-atherogenic role. In fact, previous studies have identified hypovitaminosis D as an independent predictor of cardiovascular mortality and cardiovascular events in large cohorts of healthy subjects [13].

However, approximately 85%–90% of circulating 25(OH)D is tightly bound to vitamin D binding protein (VDBP), representing the body reserve of inactive vitamin D [8]. VDBP is an α2-glycosylated globuline [9], that presents three different allelic variants, defined as Gc1F, Gc1S and Gc2 [10]. These allelic variants depend on two SNPs, rs7041 and rs4588 locate in exon 11 of chromosome 4 [11]. Gc1F is the ancestry variant, more common in black and Asiatic populations, while Gc1S, consequent to a replacement T → G in rs7041, is frequent in white patients. Finally, Gc2, due to the missense mutation C→ A in rs4588, is mostly present in Caucasian people [12]. The latter have been previously linked to an elevation of VDBP levels and higher affinity for 25(OH)D, thus conditioning its

bioavailability and effects and modulating the potential cardioprotective benefits of vitamin D supplementation.

The aim of the current study was to investigate in a large consecutive cohort of patients undergoing coronary angiography the relationship between rs7041 and rs4588 of Vitamin D Binding Protein, the levels of vitamin D and the prevalence and extent of CAD.

## METHODS

We included consecutive patients undergoing coronary angiography between January 2010 and January 2015 at the Azienda Ospedaliera-Universitaria, "Maggiore della Carità", Novara, Italy. Informed consent was obtained from all patients before angiography. The protocol was approved by our local Ethical Committee and is in accordance to the Declaration of Helsinki statements. All demographic and clinical data were prospectively collected in a dedicated database. Hypertension was defined as a systolic blood pressure (BP) > 140 mmHg and/or a diastolic BP > 90 mmHg or on-treatment with antihypertensive medications. The diagnosis of diabetes was based on previous history of diabetes treated with or without drugs, fasting glycaemia > 126 mg/dl or glycosylated haemoglobin > 6.5%. Hypercholesterolemia was defined as previous history of hypercholesterolemia, chronic treatment with any cholesterol-lowering agent at admission or fasting total cholesterol > 200 mg/dl.

### *Biochemical measurements*

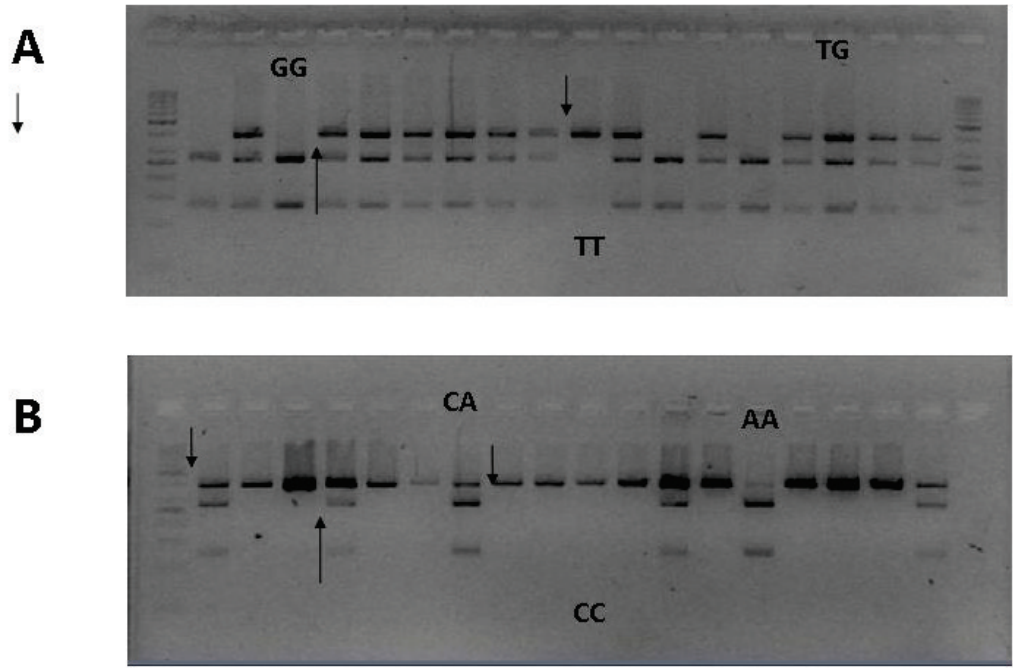
Blood samples were drawn at admission from patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography for main chemistry and genetic assessment. Glucose, creatinine, uric acid levels, blood cells count and lipid profile were determined by standard methods, as previously described [14]. 25-hydroxyvitamin D measurement was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for

25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference [15]. 25-hydroxyvitamin D inadequate levels were considered for levels below 20 ng/ml, whereas deficiency for levels < 10 ng/ml according to the US Endocrine Society guideline [15].

*VDBP polymorphisms genotyping*

Using a commercially available kit (GenElute™ Blood Genomic DNA, Sigma Aldrich) genomic deoxyribonucleic acid (DNA) was isolated from 200 ml of the peripheral blood samples. The rs7041 and rs4588 polymorphisms were studied using combined polymerase chain reaction (PCR) and restriction fragment length polymorphism technique. Following PCR conditions were used: 1.5 µl of genomic DNA were amplified in a mix containing MgCl2 [3 mM], deoxy nucleotides (dNTPs) mix [3%], 5% dimethyl sulfoxide (DMSO), 0.25 µl/100 ml DNA polymerase (GoTaq® Hot Start Polymerase, Promega) and a primers mix [3%], (where forward primer was: 5'-GAC TTC CAA TTC AGC AGC GA-3 ' and reverse: 5'-CCC TCC ACT TAA CAT GGC AG-3'), annealing temperature was settled at 60 °C.

Figure 1. Electrophoresis on High Resolution agarose gel after restriction of Polymerase Chain Reaction product with the enzymes: HaeIII (Figure 1A), showing a double electrophoretic band for homozygotes GG patients or the three bands for TG heterozygotes for rs7041 and StyI (Figure 1B) showing a double electrophoretic band for AA patients or the three bands for AC heterozygotes of rs4588 polymorphism.



The polymorphism presence was evaluated by the pattern of length of restriction fragments after digesting PCR products by HaeIII (Thermo ScientificBsuRI) and StyI (Thermo Scientific Eco130I).

In case of substitution between aspartic acid (T) and glutamine (G) in rs7041, HaeIII recognised a restriction site resulting in products of 271 and 132 base pairs, while in rs4588 the replacement between threonine (C) and lysine (A) generated a restriction site for StyI, resulting in two fragments of 278 and 125 base pairs. The digestion product was analysed with electrophoretic run on high resolution agarose 2.15% TAE gel (Figure 1 A and B).

*Coronary angiography*

Coronary angiography was routinely performed by the Judkins technique, preferring the radial approach, using 6-French right and left heart catheters. Quantitative coronary angiography was performed by two experienced interventional cardiologists [16] who had no knowledge of the patients' genetic data, by an automatic edge-detection system (Siemens Acom Quantcor QCA, Erlangen, Germany). After the visual inspection of the coronary artery, the frame of optimal clarity was selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, the analysed arterial segment with coronary lesion was defined by moving the cursor from the proximal to the distal part of coronary artery to ensure adequate determination of reference diameter. We have measured minimal luminal diameter, reference diameter, percent diameter stenosis and length of the lesion.

Significant CAD was defined as at least 1 coronary stenosis > 50%. Severe multivessel disease was defined as three-vessel disease and/or left main disease. In case of patients who had previously undergone percutaneous coronary intervention, even though no restenosis was observed, the treated vessel was counted as significantly diseased. In previously bypassed patients, native arteries

and grafts were taken into account in the evaluation of extension of artery disease (number of diseased vessels).

### *Statistical analysis*

Statistical analysis was performed using SPSS 22.0 statistical package. Continuous data were expressed as mean ± SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. The normality of the distribution was assessed by the Kolmogorov-Smirnov test. Patients were grouped according to genetic status of VDBP, performing a separate analysis for rs7041 and rs4588. For the latter SNP, carriers of the mutated allele either in heterozygosis and homozygosis were considered together. Multivariate logistic regression analysis was performed to evaluate the relationship between VDBP rs7041 polymorphism and rs4588 and coronary artery disease, after correction for baseline confounding factors that were entered in the model in block. Results were considered statistically significant for a two-tailed $p < 0.05$.

## RESULTS

We included in our study 1080 patients, among them 57% carried the mutated G allele of rs7041, 355 (32.9%) in homozygosis whereas 433 (22%) carried the A allele of rs4588, with 43 (3.98%) homozygotes. The results were in line with the expected prevalence of the mutated alleles in Caucasian population and respected the Hardy-Weinberg equilibrium ($p=0.15$ for rs7041 and $p=0.11$ for rs4588).

Baseline characteristics according to the polymorphisms are shown in Table 1.

No significant difference was observed in terms of main demographic and clinical characteristics according in carriers of the different allelic variants of VDBP, but for the observation of higher levels of C reactive protein for carriers of the G allele of rs7041 ($p=0.02$).

Table 1 Demographic, clinical characteristics, therapy at admission and baseline chemistry for rs7041 and rs4588 polymorphisms

| Variable | rs7041 | | | p-value | rs4588 | | p-value |
|---|---|---|---|---|---|---|---|
| | TT= 199 | TG= 526 | GG= 355 | | CC= 647 | AC+AA= 433 | |
| **Demographic and clinical characteristics** | | | | | | | |
| **Age (mean ± DS)** | 67.66(±10.37) | 68.39(±11.29) | 67.31(±11.59) | 0.35 | 67.94 (±11.19) | 67.82 (±11.29) | 0.86 |
| **Male gender (%)** | 73.4 | 67.4 | 68.5 | 0.34 | 67.6 | 70.8 | 0.28 |
| **Diabetes (%)** | 38.4 | 32.4 | 33.4 | 0.34 | 33.8 | 33.9 | 1.00 |
| **BMI (mean ± DS)** | 27.59 (± 4.7) | 26.97 (± 4.49) | 27.38 (±4.93) | 0.20 | 27.26 (± 4.78) | 27.16 (± 4.54) | 0.72 |
| **Hypercholesterolemia (%)** | 57.3 | 50.5 | 56.4 | 0.82 | 54.1 | 53.1 | 0.76 |
| **Smoking (%)** | | | | 0.13 | | | 0.76 |
| **Active smoking** | 16.6 | 20.9 | 25.1 | | 21.8 | 21.1 | |
| **Previous smoking** | 23.1 | 22.8 | 24.0 | | 23.5 | 23 | |
| **Systolic Blood Pressure (%)** | 74.4 | 73.2 | 70.7 | 0.32 | 72.7 | 72.3 | 0.89 |
| **Renal Insufficiency (%)** | 24.6 | 21.0 | 20.6 | 0.32 | 21.4 | 21.8 | 0.88 |
| **Previous MI(%)** | 22.6 | 22.9 | 19.8 | 0.36 | 21.1 | 22.8 | 0.55 |
| **Previous PCI (%)** | 26.3 | 21.4 | 24.3 | 0.82 | 23.6 | 22.8 | 0.77 |
| **Previous CABG (%)** | 9.5 | 10.2 | 8.9 | 0.73 | 9.7 | 9.6 | 1.00 |
| **Previous CVA (%)** | 3.5 | 8.3 | 5.0 | 0.86 | 6.4 | 6.2 | 0.89 |
| **Indication for angiography** | | | | 0.66 | | | 0.27 |
| **Stable angina (%)** | 30.8 | 22.1 | 23.0 | | 22.1 | 26.8 | |
| **DCM or valvular disease (%)** | 22.0 | 19.5 | 18.1 | | 19.5 | 19.4 | |
| **Acute Coronary Syndrome (%)** | 47.3 | 58.5 | 58.9 | | 58.4 | 53.8 | |
| **Therapy at admission** | | | | | | | |
| **Statins (%)** | 54.8 | 44.4 | 49.3 | 0.45 | 46.7 | 49.8 | 0.35 |
| **ASA (%)** | 55.8 | 53.1 | 58.0 | 0.45 | 56.1 | 53.9 | 0.50 |
| **Nitrates (%)** | 31.5 | 29.2 | 30.8 | 0.98 | 29.6 | 30.9 | 0.68 |
| **Beta-blockers (%)** | 52.8 | 50.8 | 49.9 | 0.52 | 49.8 | 52.3 | 0.46 |
| **ACE-inhibitors (%)** | 33.5 | 34.0 | 38.2 | 0.21 | 36.6 | 33.4 | 0.30 |
| **Angiotensin-receptor blockers (%)** | 28.9 | 21.6 | 21.3 | 0.07 | 23.1 | 22.6 | 0.88 |
| **Diuretics (%)** | 32.5 | 29.9 | 28.9 | 0.40 | 29.7 | 30.6 | 0.74 |
| **Ca-antagonists (%)** | 19.3 | 19.7 | 17.9 | 0.62 | 19.3 | 18.7 | 0.81 |
| **Clopidogrel (%)** | 15.7 | 15.4 | 19.9 | 0.14 | 18.2 | 15.2 | 0.22 |

*MI = myocardial infarction, PCI= percutaneous coronary intervention; CABG=coronary artery bypass grafting, CVA= cerebrovascular accidents; LDL= low-density lipoproteins; HDL=high density lipoproteins, DCM = dilated cardiomiopathy. ASA= acetylsalicylic acid; ACE= angiotensin-converting enzyme; DCM = Dilated Cardiomyopathy; WBC = White Blood Cell; RBC = Red Blood Cell*

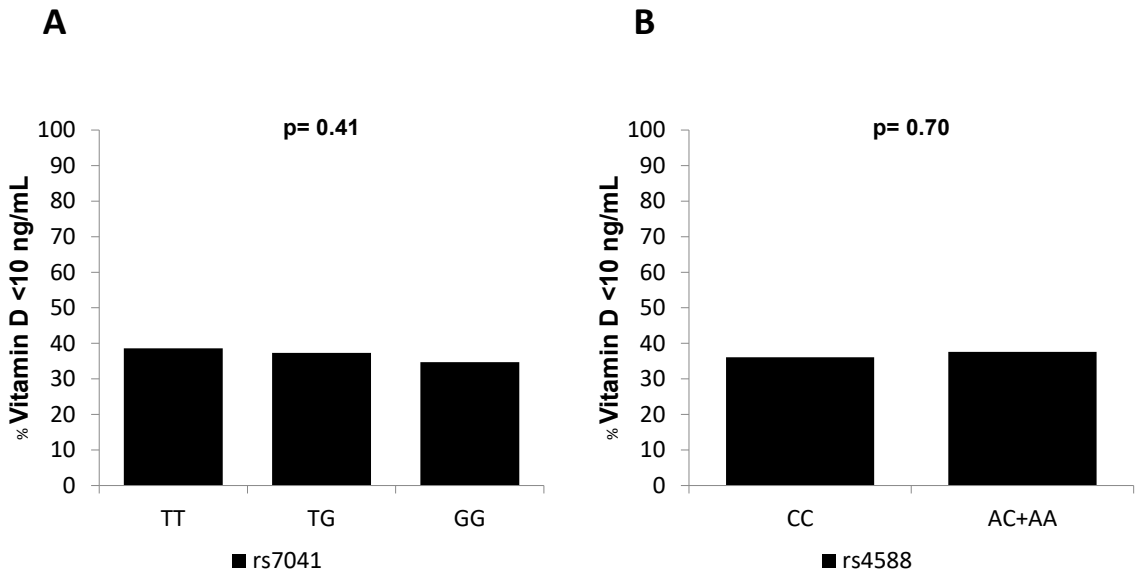
Table 2 Season values of Vitamin D according to genetic status of VDBP

| Variable | Summer Mean ± SD | p-value | Winter Mean ± SD | p-value | p-value (intergroup) |
|---|---|---|---|---|---|
| **rs7041** | | | | | <0.001 |
| TT | 19.26 ± 11.3 | | 13.8 ± 8.4 | | |
| TG | 16.89 ± 9.9 | 0.19 | 13.6 ± 12.6 | 0.86 | |
| GG | 18.40 ± 11.1 | | 14.1 ± 9.7 | | |
| **rs4588** | | | | | <0.001 |
| CC | 17.9 ± 11.0 | 0.88 | 14.3 ± 9.6 | 0.16 | |
| AC+AA | 17.7 ± 9.9 | | 13.2 ± 7.7 | | |

*VDBP polymorphisms and vitamin D*

The prevalence of inadequate levels of vitamin D was 63.5% (686 patients), with 31.9% of patients displaying deficiency (< 10 ng/ml) of 25-hydroxyvitamin D. As shown in Figure 2, genetic status of VDBP did not affect the prevalence of hypovitaminosis D (rs7041: 38.6% TT vs 37.3% TG vs 34.7% GG, p=0.41; Figure 2.A; rs4588: 36.1% CC vs 37.6% AC+AA, p=0.70; Figure 2.B)

Figure 2. Polymorphisms rs7041(A) and rs4588 (B) and prevalence of severe deficiency of 25-hydroxyvitamin D.



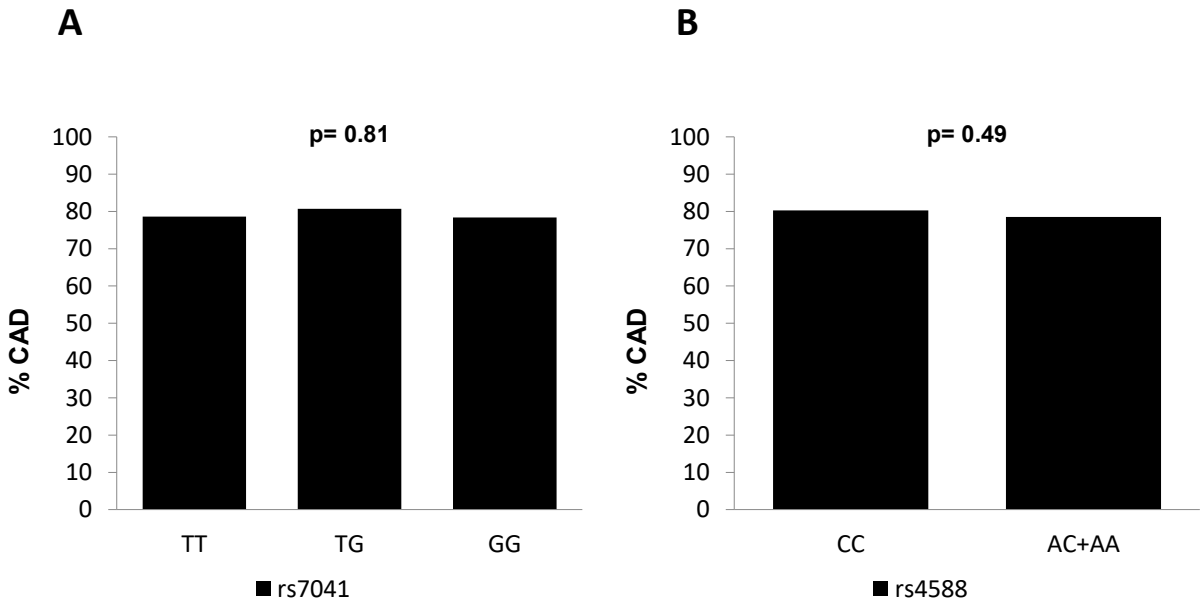
As expected, we found a significant seasonal variability between 25-hydroxyvitamin D level in winter and summer (17.8±10.8 vs 14.4±9.4; p<0.001). Nevertheless, these variations were not conditioned by genetic status (Table 2).

*VDBP polymorphisms and angiographic results*

Table 3 displays main angiographic findings (per lesion evaluation) according to VDBP genotypes. Higher prevalence of lesions in LAD and a longer lesion length were observed in rs4588 "A" carriers (p=0.04 e p=0.03 respectively). On the contrary, higher prevalence of bifurcation lesions and chronic occlusions were observed in "G" carriers (p=0.002 and p=0.01 respectively).

However, neither rs7041 nor rs4588 polymorphisms did affect the prevalence of CAD (rs7041: 79.1% TT vs 80.3% TG vs 78.5% GG, p=0.81 - Figure 3. A; rs4588= 80.3% CC vs 78.5% AC+AA, p=0.49 - Figure 3. B).

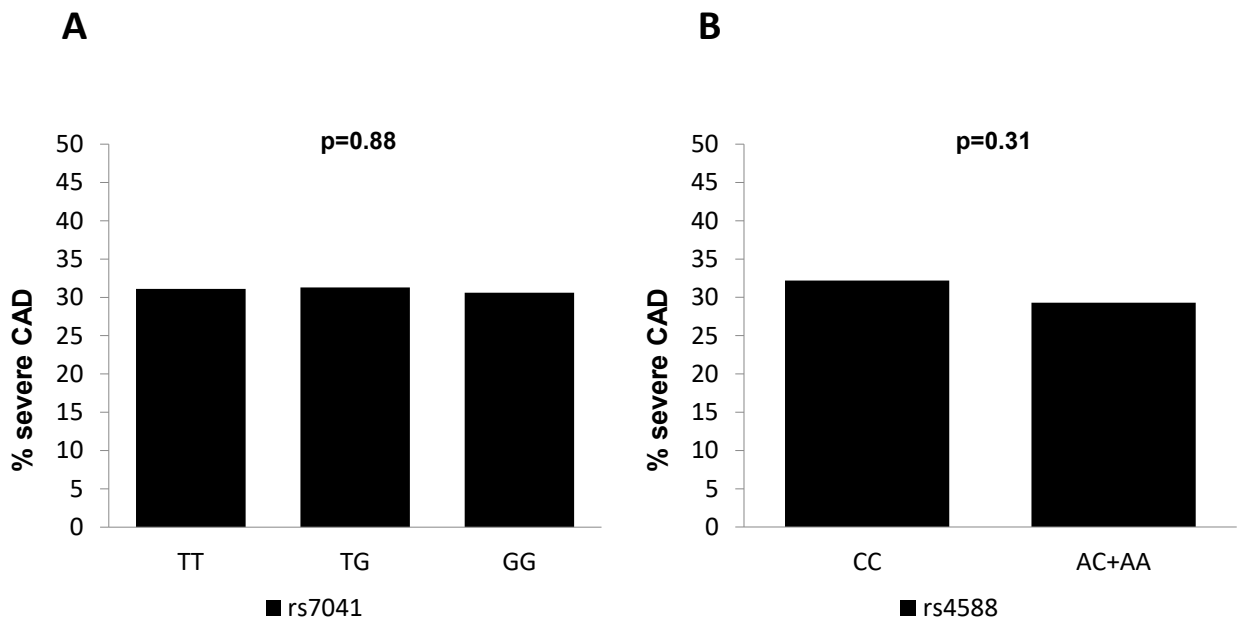
Figure 3. Polymorphisms rs7041 (A) and rs4588 (B) and prevalence of coronary artery disease (CAD).




Similar data were observed when the prevalence of severe CAD (defined as left main and/or or 3-vessel disease) was analyzed, for rs7041 (31.1% TT % vs 31.3% TG vs 30.6% GG, p=0.88) and for rs4588 (32.2% CC vs 29.3% AC+AA, p=0.31), as shown in Figure 4. A and 4. B.

Results were confirmed after adjustment for baseline confounding factors (level of C-reactive protein) for rs7041 in heterozygotes (adjusted OR [95% CI] = 1.04 [0.68-1.61], p=0.85) and homozygotes (adjusted OR GG [95% CI] = 1.13 [0.81-1.6], p=0.47); and for rs4588 (adjusted OR [95% CI] = 0.90 [0.66-1.21] p=0.49 for A allele carriers).

Figure 4. Polymorphisms rs7041 (A) and rs4588 (B) and prevalence of severe CAD.




No impact of VDBP genetics was found also for severe CAD at multivariate analysis for rs7041 (TG: OR [95% CI] = 1.06 [0.72-1.55], p=0.78; GG: OR [95% CI] =0.99 [0.74-1.33], p=0.96) and for rs4588 (AC+AA: OR [95% CI] =0.87 [0.67-1.14], p=0.31).

Besides, when including the levels of circulating 25-hydroxyvitamin D to the multivariate model, 25(OH)D status, but not genetic polymorphisms of VDBP resulted statistically associated to CAD (25-hydroxyvitamin D: OR [95% CI] = 0.99 [0.97-1.0], p=0.05; rs7041 TG: OR [95% CI] = 1.26 [0.73-2.19], p=0.41; rs7041 GG: OR [95% CI] = 1.25 [0.82-1.91], p=0.30; rs4588 AC+AA: OR [95% CI] = 0.76 [0.51-1.13], p=0.18). No significant interaction in the prevalence of CAD was observed between the genetic status of VDBP and severe hypovitaminosis D (< 10 ng/ml) for both rs7041 (25-hydroxyvitamin D > 10

ng/ml: 76.4% vs 80.4% for TT vs G –allele carriers, p=0.36; 25-hydroxyvitamin D < 10 ng/ml: 86.2% vs 80.2%, p=0.30 for TT vs G –allele carriers, p interaction =0.91) and rs4588 (25-hydroxyvitamin D >10 ng/ml: 82.8% vs 75% for CC vs A-carriers, p=0.02, for 25-hydroxyvitamin D < 10 ng/ml: 78.6% vs 85.2%, p=0.16 for CC vs A-carriers, p interaction=0.32).

Table 3 Angiographic Characteristics according to rs7041 and rs4588 polymorphisms.

| Variable | rs7041 | | | p-value | rs4588 | | p-value |
|---|---|---|---|---|---|---|---|
| | TT= 361 | TG= 950 | GG= 642 | | CC= 1164 | AC+AA= 789 | |
| **Lesion Location** | | | | | | | |
| **Left main disease (%)** | 9.3 | 9.3 | 7.3 | 0.36 | 7.5 | 10.4 | 0.12 |
| **LAD (%)** | 55.7 | 60.6 | 60.4 | 0.36 | 62.3 | 55.8 | 0.04* |
| **CX (%)** | 46.9 | 44.3 | 47.3 | 0.77 | 48.1 | 42.4 | 0.07 |
| **RCA (%)** | 50.0 | 45.6 | 45.1 | 0.32 | 47.1 | 44.9 | 0.50 |
| **Lesion Characteristics** | | | | | | | |
| **Type C Lesion (%)** | 55.5 | 56.4 | 51.3 | 0.13 | 53.2 | 56.4 | 0.19 |
| **Lesion length (mm)** | 22.4 (±14.8) | 22.9 (±14.5) | 21.9 (±14.2) | 0.34 | 21.9 (±13.6) | 23.4 (±15.5) | 0.03* |
| **Reference diameter (mm)** | 3.0 (±0.6) | 2.9 (±0.6) | 3.0 (±0.5) | 0.40 | 3.0 (±0.6) | 2.9 (±0.6) | 0.44 |
| **Percent stenosis (mean ± DS)** | 87.1 (±12.7) | 86.6 (±12.8) | 85.6 (±12.8) | 0.16 | 86.1 (±13) | 86.7 (±12.4) | 0.30 |
| **Calcifications (%)** | 17.5 | 20.6 | 14.5 | 0.09 | 18.9 | 16.6 | 0.21 |
| **Bifurcations (%)** | 15.3 | 20.3 | 23.5 | 0.002* | 21.5 | 19.0 | 0.19 |
| **Dissections (%)** | 0.0 | 0.0 | 0.2 | 0.15 | 0.1 | 0.0 | 0.28 |
| **Thrombus (%)** | 6.1 | 5.2 | 4.1 | 0.16 | 4.6 | 5.6 | 0.34 |
| **Chronic occlusion (%)** | 17.2 | 15.7 | 11.9 | 0.01* | 14.5 | 15.0 | 0.80 |
| **In-stent Restenosis (%)** | 6.4 | 3.8 | 4.8 | 0.40 | 4.9 | 4.2 | 0.51 |
| **Proximal vessel tortuosity (%)** | 0.8 | 1.6 | 0.8 | 0.67 | 1.0 | 1.5 | 0.30 |
| **TIMI flow** | | | | 0.1 | | | 0.37 |
| **3** | 71.3 | 72.9 | 76.6 | | 74.1 | 73.6 | |
| **2** | 3.6 | 4.0 | 5.1 | | 4.9 | 3.4 | |
| **1** | 2.8 | 4.0 | 2.8 | | 3.6 | 3.0 | |
| **0** | 22.3 | 19.1 | 15.5 | | 17.5 | 20.0 | |

*CAD = Coronary artery disease; LAD = Left descending coronary artery; CX = Circumflex coronary artery; RCA = Right coronary artery; TIMI = thrombolysis in myocardial infarction*

*DISCUSSION*

This is one of the largest studies conducted so far to evaluate the relationship between VDBP polymorphisms and the prevalence and extent of CAD. The main finding is that these polymorphisms are not associated with the prevalence and extent of CAD. Coronary artery disease still represents the leading cause of mortality in developed countries [1] and large interests have been focused on the identification of new biomarkers [16,17,18], that may be associated with coronary atherosclerosis. Particular attention has been focused on single nucleotide polymorphisms (SNPs), common genetic variants occurring in more than 1% of the population, whose potential role in predicting the risk of CAD and myocardial infarction has recently been pointed out in large genomic-wide association studies [19].

A raising attention, nevertheless, has been addressed to vitamin D deficiency, due to its high prevalence in the world, ranging from 13 to more than 50% of healthy subjects in western countries, according to different studies. In addition, vitamin D has been identified with a relevant implication in cardiovascular health [13,15,20]. In fact, 25-hydroxyvitamin D is a hormonal precursor modulating calcium and bone homeostasis, but also involved in the pathogenesis of CAD.

The NHANES III study showed an increased occurrence of angina, myocardial infarction and heart failure in subjects with vitamin D levels < 20 ng/mL [21] and similar results have been achieved in more recent cohort studies documenting an association between vitamin D deficiency and angiographic definition of CAD [22] or platelet reactivity [23]. Nevertheless, contrasting data have been reported so far on the potential benefits of vitamin D supplementation on cardiovascular outcomes [24-26].

Indeed, previous reports have suggested that the variations in the response to the treatment with vitamin D could be dependent on the role of Vitamin D Binding Protein (VDBP), that is the major

transporter of the vitamin in the bloodstream. Moreover, this protein also allows the megalin-mediated renal re-uptake of the complex Vitamin D-VDBP in the tubules, [27] therefore conditioning the pool of vitamin D, the levels of the free active form and its biological effects [9, 28]. Previous studies [12] have documented how Gc1S and Gc2 variants are associated with higher levels of VDBP, and then with a larger pool of 25(OHO)D than for Gc1F variant. In effect, common genetic variations of VDBP have been reported to account for more than 80% of the variability of the circulating protein and for 10% of 25-hydroxyvitamin D levels [12]. In particular, the single nucleotide polymorphisms rs7041 is dependent on a T→G substitution in exon 11, that is responsible for the substitution Asp→Glu, and translates in the allelic variant Gc1S of the protein, associated with more elevated levels of VDBP and an increased affinity for vitamin D. A similar mutation is provided by rs4588, a C→A substitution falling at 12 base pairs distance from rs7041, responsible for the Gc2 allelic variant of VDBP, in linkage disequilibrium with the previous (D'=0.99). In the previous ARIC study [29], the carriers of the rs7041 substitution, genetically predisposed to low 25-hydroxyvitamin D levels, showed a slightly increased risk of stroke at a median follow-up of 20 years. However, in the same study 25-OH D levels < 17 ng/m and not the genetic status of VDBP were linked to the risk of CAD [30].

In contrast, Powe et al. showed that the T allele of rs7041 was associated to lower levels of VDBP both in black and white population, potentially increasing the quote of free bioactive 25-hydroxyvitamin D and therefore conditioning its cardioprotective effects [12].

Our study represents one of the largest cohort of patients in which we assessed the relationship between VDBP polymorphisms and the prevalence of CAD. We documented that these two polymorphisms are not associated either with 25-hydroxyvitamin D levels or with the prevalence and extent of CAD, and even the result was independent from major risk factors for atherosclerosis after multivariate adjustments.

Indeed, our findings are in contrast with the previous reports of Stakisaitis et al. [29], demonstrating that VDBP polymorphism rs7041 increased the risk of CAD (OR= 1.45, p=0.02), while rs4588 reduced this risk (OR= 0.69; p=0.03). However, the sample examined in their study was far smaller (154 patients with CAD and 306 controls), and then, conclusion could have been conditioned by insufficient statistical power. In fact, no impact of VDBP genetics on CAD was documented in the larger ARIC study, even though the definition of coronary atherosclerosis did not require the mandatory angiographic assessment in all patients.

Analogous conclusion had also been reached in the IMPROVE [31] study, where the rs7041 and rs4588 conditioned only 25-hydroxyvitamin D levels and not markers of early atherosclerosis as the carotid intima-media thickness.

However, in our study we found an association between the G allele of rs7041 and the levels of C-reactive protein, that is considered a marker of the activation of those inflammatory processes, involved in the progression of atherosclerotic plaque [32]. Thus, it could be hypothesized that carriers of the “G” allele might display a faster or more aggressive development of CAD as compared to other genetic variants of VDBP. In fact, we also observed an association of these allelic variants with the complexity of angiographic findings, since the “G” allele of rs7041 related with the rate of lesions involving a bifurcation, whereas rs4588 was associated with lesions length.

Indeed, VDBP has directly involved in the modulation of the immune processes, favouring the chemotaxis of neutrophils, the pro-oxidative metabolism of macrophages and controlling the activation of T lymphocytes [32,33].

Nevertheless, pathophysiological mechanisms of this potential interaction are yet to be determined and we cannot exclude that these associations can represent occasional findings rather than real clinical associations. Therefore, larger studies with a prospective follow-up should be helpful in order

to clear out the role of rs7041 variants in the progression of CAD and provide potential therapeutic implications for the indications to 25-hydroxyvitamin D supplementation. In fact, the recent randomized study by Scragg et al. did not show any benefit with a monthly dose of vitamin D on cardiovascular events at an average 3-years follow-up, although different findings could have been achieved when applying different strategies of supplementation and if accounting for the genetic variants of the proteins involved in vitamin D homeostasis [34].

*Study limitations*

Limitations of the present study include those inherent to any prospective but observational study. In fact, the lower prevalence of the polymorphisms, in addition to the unbalance between the two populations, might have reduced the statistical power of our study. In addition, the prevalence of hypovitaminosis D in our population was high, as we enrolled patients with established or high suspect of cardiovascular disease and displaying several cardiovascular risk factors, such as age, diabetes or renal failure, that represent factors favoring vitamin D deficiency. Indeed, a case-control study would have overcome this limitation, however, we performed such patients' recruitment on purpose. In fact, by including a prospective cohort of patients undergoing coronary angiography, we could overcome a bias due to a potential patient selection, when they are retrospectively identified. Moreover, as coronary angiography still represents the gold standard technique to evaluate the presence and extent of CAD, we could not include a healthy control group in our study. In fact, the absence of symptoms would not have excluded with certainty the absence of coronary atherosclerosis, especially among elderly and diabetic patients, that represented a large proportion of our real-life population.

We did not collect follow-up data, especially in patients undergoing coronary angioplasty, and thus cannot exclude an impact of these polymorphisms of the progression of CAD, occurrence of acute MI and risk of adverse events after PCI. In fact, we observed an association of rs7041 with the levels of

C reactive protein, that have been proposed as a marker of vascular wall damage, potentially conditioning the evolution of atherosclerotic plaque.

A further limitation could be the absence of VDBP levels dosing, which did not make possible the evaluation of real free vitamin D, and also the paucity of patients receiving vitamin D supplementation, not allowing a separate analysis for this subgroup of patients.

## CONCLUSION

This study showed in a large cohort of patients undergoing coronary angiography, that the polymorphisms rs7041 and rs4588 of VDBP are not associated with the levels of 25-hydroxyvitamin D nor with the prevalence and extent of CAD. In fact, 25-hydroxyvitamin D levels but not VDBP genetic status independently predicted the occurrence of coronary lesions at angiography.

Thus, until the results of larger studies become available, these polymorphisms cannot be considered risk factors for coronary and carotid atherosclerosis.

## BIBLIOGRAPHY


1. Antman EM, Hand M, Armstrong PW, Bates ER, Green LA, Halasyamani LK, Hochman JS, Krumholz HM, Lamas GA, Mullany CJ, Pearle DL, Sloan MA, Smith SC Jr; 2004Writing Committee Members., Anbe DT, Kushner FG, Ornato JP, Jacobs AK, Adams CD, Anderson JL, Buller CE, Creager MA, Ettinger SM, Halperin JL, Hunt SA, Lytle BW, Nishimura R, Page RL, Riegel B, Tarkington LG, Yancy CW. 2007 Focused update of the ACC/AHA 2004 guidelines for the management of patients with ST-elevation myocardial infarction: A report of the American College of Cardiology/American Heart Association task force on practice guidelines. Circulation. 2008;117:296-329.
2. De Luca G, Cassetti E, Marino P. Percutaneous coronary intervention-related time delay, patient's risk profile, and survival benefits of primary angioplasty vs lytic therapy in ST-segment elevation myocardial infarction. Am J Emerg Med. 2009;27:712-719.
3. De Luca G, Suryapranata H, Marino P. Reperfusion strategies in acute ST-elevation myocardial infarction: an overview of current status. Prog Cardiovasc Dis. 2004;50:352-382.
4. De Luca G, Gibson CM, Bellandi F, Noc M, Dudek D, Zeymer U, Arntz HR, Cutlip D, Maioli M, Zorman S, Mesquita Gabriel H, Emre A, Rakowski T, Gyongyosi M, Huber K, Van't Hof AW. Diabetes mellitus is associated with distal embolization, impaired myocardial perfusion, and higher mortality in patients with ST-segment elevation myocardial infarction treated with primary angioplasty and glycoprotein IIb-IIIa inhibitors. Atherosclerosis. 2009;207:181-5.
5. De Luca G, van 't Hof AW, Ottervanger JP, Hoorntje JC, Gosselink AT, Dambrink JH, de Boer MJ, Suryapranata H. Ageing, impaired myocardial perfusion, and mortality in patients with ST-segment elevation myocardial infarction treated by primary angioplasty. Eur Heart J. 2005;26:662-6.
6. De Luca G, Ernst N, van 't Hof AW, Ottervanger JP, Hoorntje JC, Gosselink AT, Dambrink JH, de Boer MJ, Suryapranata H. Predictors and clinical implications of early reinfarction after primary angioplasty for ST-segment elevation myocardial infarction. Am Heart J. 2006;151:1256-9.
7. Mancia G, Mancia G, Fagard R, Narkiewicz K, Redon J, Zanchetti A, Böhm M, Christiaens T, Cifkova R, De Backer G, Dominiczak A, Galderisi M, Grobbee DE, Jaarsma T, Kirchhof P, Kjeldsen SE, Laurent S, Manolis AJ, Nilsson PM, Ruilope LM, Schmieder RE, Sirnes PA, Sleight P, Viigimaa M, Waeber B, Zannad F, Redon J, Dominiczak A, Narkiewicz K, Nilsson PM, Burnier M, Viigimaa M, Ambrosioni E, Caufield M, Coca A, Olsen MH, Schmieder RE, Tsioufis C, van de Borne P, Zamorano JL, Achenbach S, Baumgartner H, Bax JJ, Bueno H, Dean V, Deaton C, Erol C, Fagard R, Ferrari R, Hasdai D, Hoes AW, Kirchhof P, Knuuti J, Kolh P, Lancellotti P, Linhart A, Nihoyannopoulos P, Piepoli MF, Ponikowski P, Sirnes PA, Tamargo JL, Tendera M, Torbicki A, Wijns W, Windecker S, Clement DL, Coca A, Gillebert TC, Tendera M, Rosei EA, Ambrosioni E, Anker SD, Bauersachs J, Hitij JB, Caulfield M, De Buyzere M, De Geest S, Derumeaux GA, Erdine S, Farsang C, Funck-Brentano C, Gerc V, Germano G, Gielen S, Haller H, Hoes AW, Jordan J, Kahan T, Komajda M, Lovic D, Mahrholdt H, Olsen MH, Ostergren J, Parati G, Perk J, Polonia J, Popescu BA, Reiner Z, Rydén L, Sirenko Y, Stanton A, Struijker-Boudier H, Tsioufis C, van de Borne P, Vlachopoulos C, Volpe M, Wood DA. 2013 ESH/ESC guidelines for the management of arterial hypertension: the Task Force for the Management of Arterial Hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). Eur Heart J. 2013;34:2159-219
8. Schneider AL, Lutsey PL, Selvin E, Mosley TH, Sharrett AR, Carson KA, Post WS, Pankow JS, Folsom AR, Gottesman RF, Michos ED. Vitamin D, vitamin D binding protein gene polymorphisms, race and risk of incident stroke: the Atherosclerosis Risk in Communities (ARIC) study. Eur J Neurol. 2015;22:1220-7.
9. Delanghe JR, Speeckaert R, Speeckaert MM. Behind the scenes of vitamin D binding protein: more than vitamin D binding. Best Pract Res Clin Endocrinol Metab. 2015;29:773-786.
10. Speeckaert M, Huang G, Delanghe JR, Taes YEC. Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. Clin Chim Acta. 2006;372:33-42.
11. Miller JR, Lechler PJ, Mackin G, Germanoski D, Villarroel LF. Evaluation of particle dispersal from mining and milling operations using lead isotopic fingerprinting techniques, Rio Pilcomayo Basin, Bolivia. Sci Total Environ. 2007;384:355-373.
12. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, Powe NR, Thadhani R. Vitamin D-binding protein and vitamin D status of black Americans and white Americans. N Engl J Med. 2013 21;369:1991-2000.
13. Kunadian V, Ford GA, Bavamia B et al. Vitamin D deficiency and coronary artery disease: A review of the evidence. Am Heart J 2014;167:283-91
14. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P; Novara Atherosclerosis Study Group (NAS). Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012;22:426-33.

15. Holick MF. Vitamin D Deficiency. N Engl J Med. 2007;357:266-281.
16. Eren E, Yilmaz N, Aydin O. Functionally defective high-density lipoprotein and paraoxonase: a couple for endothelial dysfunction in atherosclerosis. Cholesterol. 2013;2013:792090.
17. Cooney MT, Dudina AL, Graham IM. Value and Limitations of Existing Scores for the Assessment of Cardiovascular Risk: A Review for Clinicians. J Am Coll Cardiol. 2009;54:1209-1227.
18. De Luca G, Santagostino M, Secco GG, Cassetti E, Giuliani L, Franchi E, Coppo L, Iorio S, Venegoni L, Rondano E, Dell'Era G, Rizzo C, Pergolini P, Monaco F, Bellomo G, Marino P. Mean platelet volume and the extent of coronary artery disease: results from a large prospective study. Atherosclerosis. 2009;206:292-7.
19. Samani NJ, Erdmann J, Hall AS, Hengstenberg C, Mangino M, Mayer B, Dixon RJ, Meitinger T, Braund P, Wichmann HE, Barrett JH, König IR, Stevens SE, Szymczak S, Tregouet DA, Iles MM, Pahlke F, Pollard H, Lieb W, Cambien F, Fischer M, Ouwehand W, Blankenberg S, Balmforth AJ, Baessler A, Ball SG, Strom TM, Braenne I, Gieger C, Deloukas P, Tobin MD, Ziegler A, Thompson JR, Schunkert H; WTCCC and the Cardiogenics Consortium.. Genomewide association analysis of coronary artery disease. N Engl J Med. 2007 2;357:443-53.
20. Cashman KD, Dowling KG, Skrabakova Z et al. Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr. 2016;103:1033-44
21. Kendrick J, Targher G, Smits G, Chonchol M. 25-Hydroxyvitamin D deficiency is independently associated with cardiovascular disease in the Third National Health and Nutrition Examination Survey. Atherosclerosis. 2009;205:255-260.
22. Nardin M, Verdoia M, Schaffer A, Barbieri L, Marino P, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D status, diabetes mellitus and coronary artery disease in patients undergoing coronary angiography. Atherosclerosis. 2016;250:114-21.
23. Verdoia M, Pergolini P, Rolla R, Sartori C, Nardin M, Schaffer A, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, Luca GD; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets. 2016;27:576-82.
24. Norman PE, Powell JT. Vitamin D and Cardiovascular Disease. Circ Res. 2014;114:379-393.
25. Avenell A, MacLennan GS, Jenkinson DJ, McPherson GC, McDonald AM, Pant PR, Grant AM, Campbell MK, Anderson FH, Cooper C, Francis RM, Gillespie WJ, Robinson CM, Torgerson DJ, Wallace WA; RECORD Trial Group.. Long-term follow-up for mortality and cancer in a randomized placebo-controlled trial of vitamin D(3) and/or calcium (RECORD trial). J Clin Endocrinol Metab. 2012;97:614-22.
26. Zittermann A, Iodice S, Pilz S, Grant WB, Bagnardi V, Gandini S. Vitamin D deficiency and mortality risk in the general population: a meta-analysis of prospective cohort studies. Am J Clin Nutr. 2012;95:91-100.
27. Holick M. Bioavailability of Vitamin D and its metabolites in Black and white adults. New Engl J Med 2013; 369:2047-8
28. White P, Cooke N. The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab. 2000;11:320-327.
29. Michos Erin D, Misialek Jeffrey R, Selvin Elizabeth, Folsom Aaron R, Pankow James S, Post Wendy S, Lutsey Pamela L. 25-hydroxyvitamin D levels, vitamin D binding protein gene polymorphisms and incident coronary heart disease among whites and blacks: The ARIC study. Atherosclerosis. 2015;241:12-17.
30. Stakisaitis D, Lesauskaitė V, Girdauskaitė M, Janulionis E, Ulys A, Benetis R. Investigation of Vitamin D-Binding Protein Polymorphism Impact on Coronary Artery Disease and Relationship with Longevity: Own Data and a Review. Int J Endocrinol. 2016;2016:8347379.
31. Strawbridge RJ, Deleskog A, McLeod O, Folkersen L, Kavousi M, Gertow K, Baldassarre D, Veglia F, Leander K, Gigante B, Kauhanen J, Rauramaa R, Smit AJ, Mannarino E, Giral P, Dehghan A, Hofman A, Franco OH, Humphries SE, Tremoli E, de Faire U, Gustafsson S, Östensson CG, Eriksson P, Öhrvik J, Hamsten A. A serum 25-hydroxyvitamin D concentration-associated genetic variant in DHCR7 interacts with type 2 diabetes status to influence subclinical atherosclerosis (measured by carotid intima-media thickness). Diabetologia. 2014;57:1159-72.
32. Ridker PM, Stampfer MJ, Rifai N. Novel Risk Factors for Systemic Atherosclerosis. JAMA. 2001;285:2481.
33. Calabro P, Willerson JT, Yeh ETH. Inflammatory Cytokines Stimulated C-Reactive Protein Production by Human Coronary Artery Smooth Muscle Cells. Circulation. 2003;108:1930-1932.
34. Scragg R, Stewart AW, Waayer D, Lawes CM, Toop L, Sluyter J, Murphy J, Khaw KT, Camargo CA Jr. Effect of Monthly High-Dose Vitamin D Supplementation on Cardiovascular Disease in the Vitamin D Assessment Study : A Randomized Clinical Trial. JAMA Cardiol. 2017 Apr 5. doi: 10.1001/jamacardio.2017.0175. [Epub ahead of print]

# Chapter 6:

# Vitamin D Binding Protein rs7041 polymorphism and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor.

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## Abstract

**Background.** Vitamin D deficiency represents a major health problem in general population, especially for its association with cardiovascular disorders and thrombotic risk, even in patients on dual antiplatelet therapy (DAPT). Vitamin D Binding Protein (VDBP) is the main transporter of vitamin D in the bloodstream and genetic polymorphisms of this protein have been shown to account for a significant variability of vitamin D levels and its systemic effects. Contrasting data have linked the rs7041 T□G substitution with cardiovascular disease. However, no study has so far addressed the role of rs7041 polymorphism on platelet reactivity in patients on DAPT, that was the aim of the present study.

**Methods.** Patients treated with DAPT (ASA and clopidogrel or ticagrelor) for an ACS or elective PCI were scheduled for platelet function assessment at 30-90 days post-discharge. Platelet function was assessed by Multiplate® (Roche Diagnostics AG), and VDBP genetic status by polymerase chain reaction and restriction fragment length polymorphism technique. Fasting samples were obtained for main chemistry parameters and vitamin D levels assessment.

**Results.** We included 400 patients, 187 (46.8%) receiving clopidogrel and 213 (53.2%) ticagrelor. The genetic polymorphism rs7041 (T→G) was observed in 318 patients, (79.5%), in 38.7% of them in homozygosis. Main clinical and chemistry features did not significantly differ according to genetic status, but for a higher rate of ACE-inhibitors and beta-blockers use among the carriers of the G allele

(p=0.04 and p=0.01, respectively).

VDBP genetic status did not affect the rate of HRPR with ADP-antagonists (25.6% vs 24.6% vs 28.5%, p=0.59; adjusted OR[95%CI]= 0.94[0.52-1.7], p=0.83 for T/G patients; adjusted OR[95%CI]= 1.14[0.6-2.2], p=0.67 for G homozygotes).

However, the rate of HRPR with ADP-antagonists was influenced by severe hypovitaminosis D (< 10 ng/ml) only in patients carrying the G allele, especially in homozygosis (T/T: 25.9% vs 26.1%, p=0.99; G carriers: 22.1% vs 35.3%, p=0.02, $p_{interaction}$=0.019; adjusted OR[95%CI]=1.93[1.11-3.34],p=0.02 for G carriers).

Conclusion. The present study shows that rs741 polymorphism of Vitamin D Binding Protein does not affect platelet reactivity or the rate of HRPR among patients receiving DAPT. However, the carriage of the G allele could condition the impact of hypovitaminosis D on the response to antiplatelet agents, increasing the occurrence of HRPR especially in homozygotes, thus suggesting a more significant role of vitamin D deficiency among these patients.

*Keywords*: *vitamin D binding protein, vitamin D, platelet aggregation, dual antiplatelet therapy; clopidogrel, ticagrelor, coronary artery disease*

## Introduction

Optimal antithrombotic therapy and platelet inhibition represents a crucial point in the management of acute coronary syndromes (ACS), especially in patients undergoing percutaneous coronary interventions (1-3). Nevertheless, inadequate effectiveness of dual antiplatelet therapy agents has been described (4), and mainly with ADP-antagonists rather that with ASA, occurring in up to 10-30% of patients and depending on the type of drug and baseline thrombotic profile of the patient (5,6). Previous studies have shown an association between high residual platelet reactivity (HRPR) on DAPT and an increased risk of recurrent ischemic events and stent thrombosis, therefore raising the importance of addressing those factors that may predict an impaired response to antiplatelet agents (7-9).

Among clinical conditions enhancing platelet reactivity, large interests have been focused on vitamin D deficiency (10), that represents a widespread condition, often involving those more complex and frail patients that display per se an increased cardiovascular risk, especially after coronary interventions (11-14). Nevertheless, contrasting data have been reported so far on the potential benefits of vitamin D supplementation on cardiovascular endpoints, as the protective effects on atherosclerosis and thrombosis shown by vitamin D in molecular models have not been demonstrated so far in large clinical trials and meta-analysis (15-17).

Vitamin D Binding Protein (VDBP) is the major transporter of vitamin D in the bloodstream, and genetic variants of VDBP have been demonstrated to account for the 10% of the variability of circulating levels of vitamin D (18), therefore potentially conditioning its bioavailability and antiplatelet effect. In particular the rs7041 polymorphism is a missense Asp→Glu mutation, depending from a T→G substitution in position 416 of exon 11. The allelic variant with G, defined as isoform Gc1S of the protein, is more frequent in Caucasians (minor allele frequency about 44%), and

has been associated with increased levels of VDBP and higher affinity for the vitamin, thus reducing the quote of “free” bioactive vitamin D (19). However, no study has so far addressed the impact of rs7041 polymorphism of VDBP on platelet reactivity among patients on DAPT, that was therefore the aim of present study.

## Methods

We included patients admitted Division of Cardiology, “Maggiore della Carità” Hospital, Eastern Piedmont University in Novara, Italy, from October 2009 to April 2016 requiring dual antiplatelet therapy for acute coronary syndromes or after PCI for stable coronary artery disease. Invasive treatment with coronary angiography and eventual coronary stenting was not a required inclusion criterion. All patients receiving at discharge dual antiplatelet therapy with ASA (100 to 160 mg daily) and ADP-antagonists (clopidogrel 75 mg daily or ticagrelor 90 mg b.i.d) were scheduled for chemistry and platelet function tests evaluation at 30-90 days from discharge. The study was approved by our local Ethical Committee and informed consent was obtained by all patients.

Main demographic, clinical and angiographic data, together with the indication to dual antiplatelet therapy were recorded at discharge and included in a dedicated database, protected by password. As previously described (20), hypertension was defined as systolic pressure > 140 mm Hg and/or diastolic pressure > 90 mm Hg or if the individual was taking anti hypertensive medications. Diabetes mellitus was defined as previous diagnosis, specific treatment administration (oral drug or insulin), fasting glycemia > 126 mg/dL or HbA1c > 6.5%. Chronic renal failure was considered for history of renal failure or an admission glomerular filtrate (GFR) < 60 mol/min/1.73m2 by MDRD (Modification of Diet in renal Disease) formula. The study was performed conform the declaration of Helsinki.

## Biochemical measurements

Fasting blood samples were drawn from all patients for main chemistry and vitamin D levels assessment. Vitamin D dosing was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for 25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference (21). Severe vitamin D deficiency was considered for levels beyond 10 ng/ml according to literature.

## Platelet function assessment

Platelet aggregation was measured by whole blood tests, including impedance aggregometry (Multiplate®- multiple platelet function analyser; Roche Diagnostics AG) For Multiplate a whole blood sample was stored in Vacutainer standard lithium heparin tubes and analyzed within 1-2 hours from collection (22). Tests with different agonists were performed: arachidonic acid (AA), collagen, ADP and prostaglandin E1 and thrombin receptor activating peptide (TRAP-6). Results were expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). HRPR was considered for AU*min values above lower limit normal for ASA (HAPR), [range: 862 - 1344] or after ADP stimulation [range: 417 - 1030], respectively (23-24). The test was repeated in patients with HRPR to confirm the findings.

## Genetic analysis

Genomic DNA was obtained from 200 ml of whole blood through a dedicated kit (GenElute Blood Genomic DNA, Sigma Aldrich). Target region of VDBP gene was amplified by polymerase chain reaction (PCR) using following primers: 5'- GACTTCCAATTCAGCAGCGA-3' and 5'- CCCTCCACTTAACATGGCAG -3'. Genomic DNA (1.5 µl) was amplified in 23.5 µl of a mix composed by standard concentrations of reagents, including MgCl2 3mM and 5% dimethyl sulfoxide. A negative control containing no genomic DNA was added for every PCR reaction. PCR product of 403 base pairs was then digested by restriction enzyme HaeIII (Thermo Scientific BsuRI), producing two fragments of 271 and 132 bp respectively in presence of the T→G substitution of rs7041.

## Statistical analysis

Statistical analysis was performed using SPSS 22.0 statistical package. Continuous data were expressed as mean ± SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Patients were grouped according to genetic status of VDBP. Multivariate logistic regression analysis was performed to evaluate the relationship between VDBP rs7041 polymorphism and HRPR, after correction for baseline differences that were entered in the model in block. A p value < 0.05 was considered statistically significant. Linear regression analysis was performed to assess the relationship between platelet reactivity and vitamin D levels according to VDBP genetic status.

**Table 1.** Clinical characteristics according to Vitamin D Binding Protein rs 7041 status

| Baseline clinical characteristics | T/T N= 82 | T/G N= 195 | GG N= 123 | P value |
|---|---|---|---|---|
| Age (mean±SD) | 67.9±9.8 | 67.6±11 | 66.7±11.7 | 0.71 |
| Male Sex (%) | 74.4 | 73.3 | 79.7 | 0.32 |
| BMI (mean±SD) | 26.9±4 | 26.7±4.5 | 27.5±4.2 | 0.30 |
| Hypercholesterolemia(%) | 60.5 | 54.6 | 55.3 | 0.52 |
| Diabetes mellitus (%) | 30.9 | 34.9 | 35.8 | 0.69 |
| Renal failure (%) | 18.3 | 17.6 | 17.1 | 0.82 |
| Active smokers (%) | 23.2 | 22.6 | 30.1 | 0.27 |
| Hypertension (%) | 71.6 | 69.1 | 75.6 | 0.44 |
| History of MI (%) | 17.3 | 21.6 | 22 | 0.46 |
| Previous PCI (%) | 29.6 | 25.1 | 28.5 | 0.95 |
| Previous CABG (%) | 11.1 | 12.3 | 6.5 | 0.22 |
| Indication to angiography | | | | 0.12 |
| Stable angina/ silent ischemia (%) | 26.8 | 25.1 | 31.7 | |
| STEMI/ACS (%) | 61 | 68.7 | 65 | |
| Other (%) | 12.2 | 6.2 | 3.3 | |
| Concomitant medications | | | | |
| ACE inhibitors(%) | 37.8 | 52.3 | 53.7 | 0.04 |
| ARB (%) | 26.8 | 18.5 | 22.8 | 0.63 |
| Beta blockers (%) | 64.6 | 75.9 | 80.5 | 0.01 |
| Nitrates (%) | 43.9 | 39.5 | 44.7 | 0.80 |
| Statins (%) | 70.7 | 79.5 | 80.5 | 0.13 |
| Calcium antagonists (%) | 18.3 | 20 | 22.8 | 0.42 |
| Diuretics (%) | 39 | 26.7 | 30.9 | 0.32 |

*CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers;*

## Results

Our population in represented by 400 patients, receiving DAPT (ASA/Clopidogrel in 187 patients (46.8%) and ASA/Ticagrelor in 213 patients (53.2%). Among them, 13 patients were receiving vitamin D supplementation. The genetic polymorphism rs7041 (T□G) was observed in 318 patients, (79.5%), in 38.7% of them in homozygosis.

As shown in Table 1 patients did not differ significantly for main clinical and demographic features according to genetic status, but for a higher rate of ACE-inhibitors and beta-blockers use among the carriers of the G allele (p=0.04 and p=0.01, respectively).

VBDP polymorphism did not influence main chemistry parameters, including vitamin D levels and mean platelet reactivity, as displayed in Table 2.

**Table 2.** Biochemistry parameters according to Vitamin D Binding Protein rs 7041 status

| Biochemistry parameters | T/T | T/G | GG | P value |
|---|---|---|---|---|
| Platelets (10^6/ml; mean± SD) | 241.1±78.4 | 235.4±66.6 | 228.2±66.8 | 0.41 |
| Haemoglobin (g/dl) | 13.6±1.8 | 13.3±1.6 | 13.3±1.7 | 0.21 |
| WBC (10^3/ml;mean± SD) | 7.9±2.2 | 7.9±3 | 8±2.2 | 0.99 |
| HDL cholesterol (mg/dL) | 43.6±13.4 | 42.5±15.9 | 44.3±16.2 | 0.61 |
| LDL cholesterol (mg/dl) | 80.9±35.9 | 77.4±29.2 | 76.3±32.7 | 0.58 |
| Glycaemia (mg/dL) | 124.1±34 | 121.4±50.1 | 119.3±35.6 | 0.73 |
| Glycosylated hemoglobin (%) | 6.4±1.2 | 6.2±1.1 | 6.4±1.3 | 0.18 |
| Creatinine (mg/dL) | 0.98±0.33 | 1.04±0.72 | 1.04±0.59 | 0.71 |
| C reactive protein (mg/dL) | 0.59±1.07 | 0.65±1.04 | 0.83±2 | 0.44 |
| Vitamin D (ng/ml) | 17.2±9.6 | 17.4±9.9 | 18±11.6 | 0.84 |
| ASPI test (AU*min) | 328.6±189 | 340.9±187.2 | 362.5±230.3 | 0.46 |
| COL test (AU*min) | 471.6±151 | 461.1±147.6 | 470.8±166.3 | 0.81 |
| TRAP test (AU*min) | 1217±349 | 1120.2±317 | 1116.9±342 | 0.47 |
| ADP test (AU*min) | 345.8±199.4 | 322.2±178.6 | 347.2±180.3 | 0.41 |

No difference was observed in the rate of HRPR with ASA (HAPR), as displayed in Figure 1 (2.4% vs 1.5% vs 4.1%, p=0.37).

VDBP genetic status did not also affect the rate of HRPR with the ADP-antagonists clopidogrel and

ticagrelor (25.6% vs 24.6% vs 28.5%, p=0.59); Figure 2. Results were confirmed at multivariate analysis after correction for baseline differences (adjusted OR[95%CI]= 0.94[0.52-1.7], p=0.83 for T/G patients; adjusted OR[95%CI]= 1.14[0.6-2.2], p=0.67 for G homozygotes).

Similar findings were observed in clopidogrel treated patients (34.8% vs 40.2% vs 40.7%, p=0.56) and with ticagrelor (13.9% vs 12.4% vs 17.2%, p=0.55) and when excluding the patients receiving vitamin D supplementation (24.1% vs 24.6% vs 28.9%, p=0.40).

Patients with severe hypovitaminosis D (<10 ng/ml) displayed a significantly higher degree of HRPR with ADP antagonists (33.9% vs 22.3%, p=0.02).

However, a significant interaction was observed between VDBP polymorphism and vitamin D levels, affecting platelet reactivity. In fact, as shown in Figure 3, the rate of HRPR with ADP-antagonists was influenced by hypovitaminosis D (< 10 ng/ml; n=108 patients, 27%) only among patients carrying the G allele, (T/T: 25.9% vs 26.1%, p=0.99; G carriers: 22.1% vs 35.3%, p=0.02; $p_{interaction}$ =0.019); and especially in homozygotes (respectively T/G: 21.4% vs 30%, p=0.25; G/G: 23.3% vs 42.9%, p=0.05).
The increased rate of HRPR with ADP-antagonists in patients with hypovitaminosis D and G allele of rs7041 polymorphism was confirmed at multivariate analysis (T/T patients: adjusted OR[95%CI]=1.01[0.33-3.05], p=0.99; G carriers: adjusted OR[95%CI]=1.93[1.11-3.34]; p=0.02 for severe vitamin D deficiency).
Similar results were obtained at linear regression analysis, when considering values of vitamin D as a continuous variable (T/T: r=0.12, p=0.29 and G carriers: r=-0.18, p=0.001), as shown in Figure 4 A and B respectively.

Figure 1 Bar graph shows the prevalence of high-residual on treatment platelet reactivity (HRPR) for Acetylsalicylic acid (ASA) according to vitamin D Binding protein rs7041 genetic status.

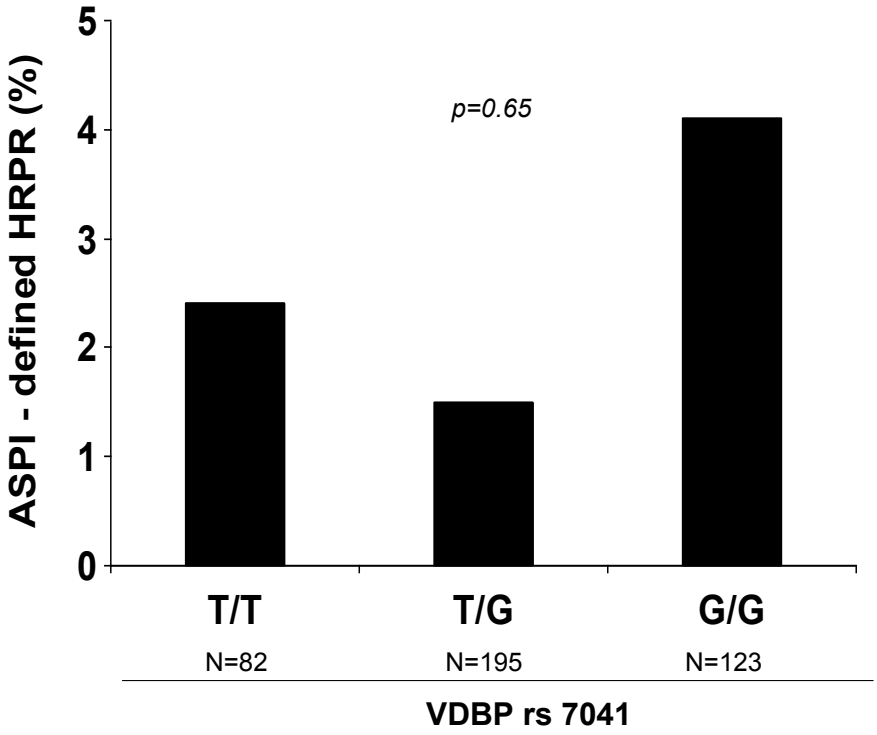



Figure 2 Bar graph shows the prevalence of high-residual on treatment platelet reactivity (HRPR) at ADP test according to vitamin D Binding protein rs7041 genetic status

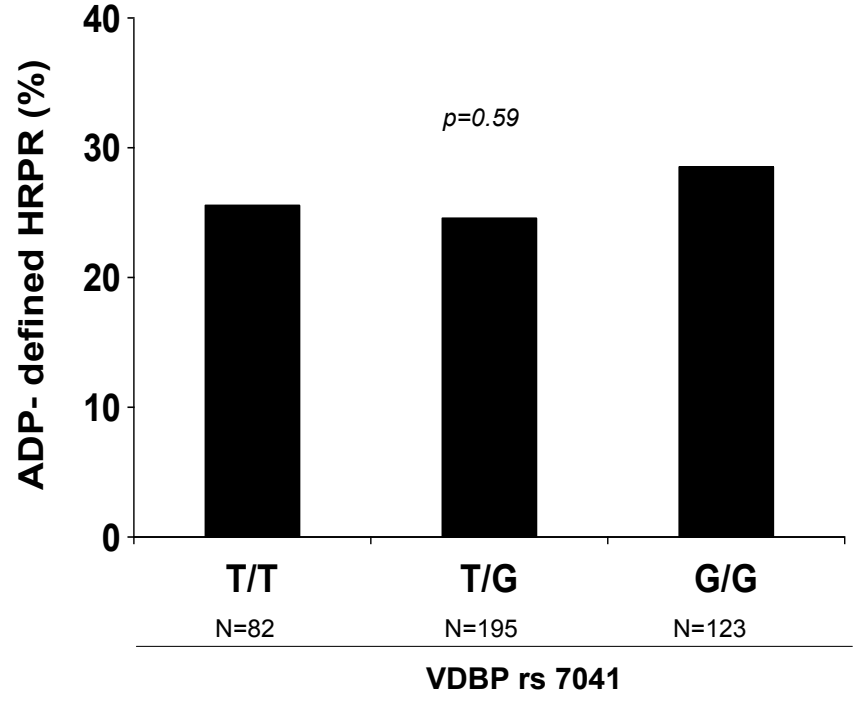


Figure 3 Bar graphs show the prevalence of high-residual on treatment platelet reactivity (HRPR) at ADP test according to vitamin D levels (below or equal/above 10 ng/ml) and vitamin D Binding protein rs7041 genetic status

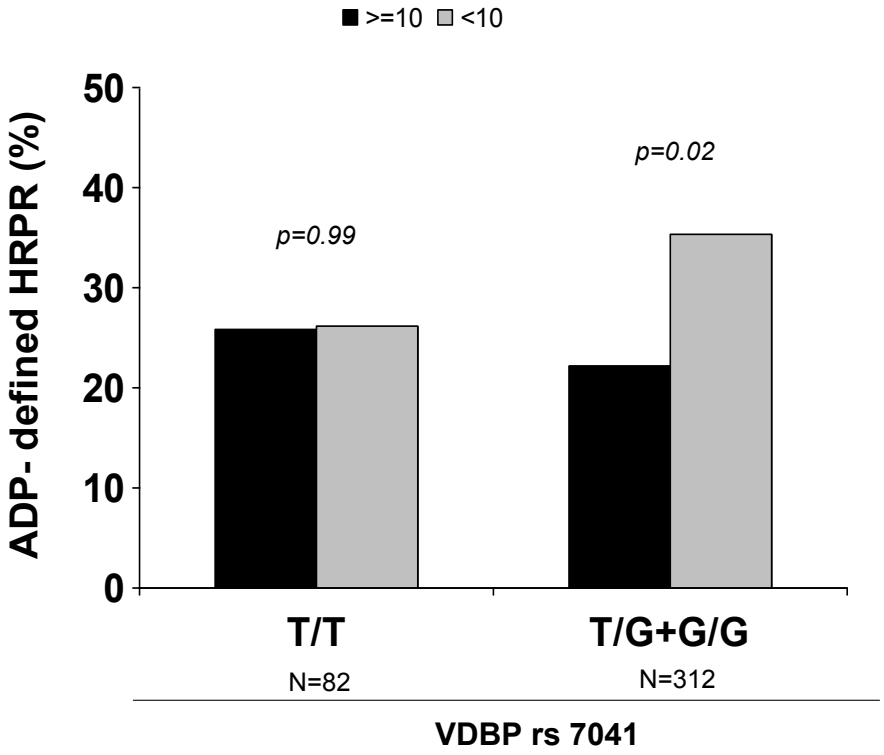
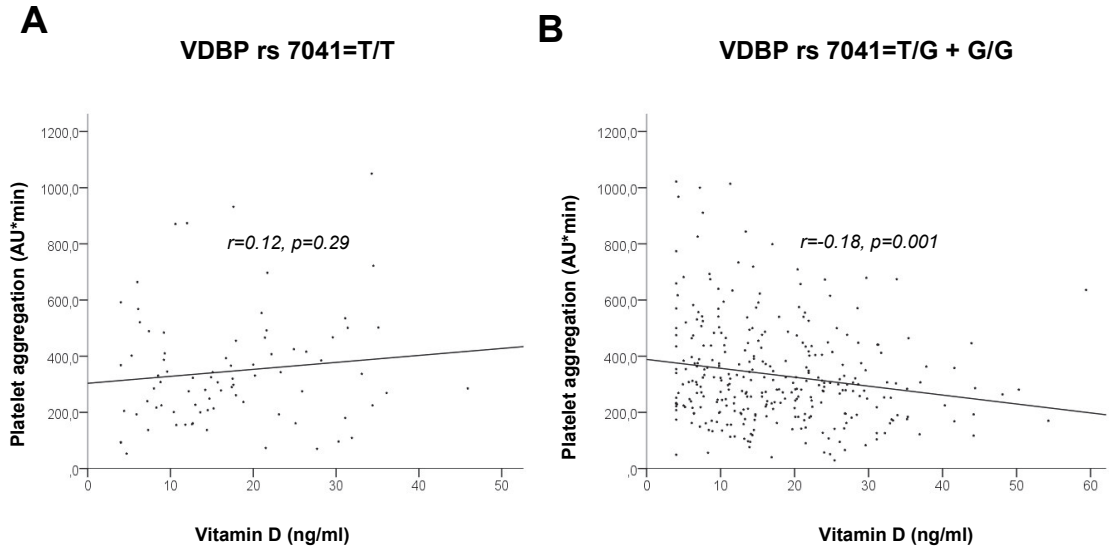



Figure 4 Linear regression analysis of platelet ADP-mediated aggregation and vitamin D levels according to vitamin D Binding protein rs7041 genetic status.




No interaction (p =0.49) was observed between clopidogrel and ticagrelor in the rate of HRPR with hypovitaminosis D and G allele (G carriers: 40.7% vs 37.7%, p = 0.80, in clopidogrel treated patients; 16.7% vs 10.5%, p=0.48, in ticagrelor treated patients).

## Discussion

The present study represents the first attempt to define the role of rs7041 polymorphism of Vitamin D Binding protein (VDBP) on platelet aggregation in vivo, in patients receiving dual antiplatelet therapy for a recent ACS or PCI.

Our main finding is the lack of an overall effect of this genetic variant on platelet reactivity or the effectiveness of antiplatelet agents. However, we observed a significant interaction between vitamin D levels and this polymorphism, as vitamin D deficiency was associated with a suboptimal platelet inhibition in response to ADP-antagonists only in the carriers of the G allele, especially in homozygosis.

Even though a unique consensus on the definition and evaluation of high-on treatment platelet reactivity (HRPR) is still lacking, the achievement of an adequate level of platelet inhibition is universally accepted as a key point in the management of patients with acute cardiovascular events, especially in case of stent implantation (24-26).

In fact, conditions enhancing platelet reactivity have been associated with an up to nine-times increased risk of recurrent ischemic events or stent thrombosis, although the underlying mechanisms have been poorly defined so far (27-29).

Clinical conditions as far as genetics (30-31), have been involved in the suboptimal effectiveness of clopidogrel, with genetic polymorphisms of the cytochrome P450 accounting for a reduced transformation of the drug into its active form and therefore a lower platelet inhibition. However, HRPR has been reported in about 10% of patients even with more recently developed antiplatelet drugs as Ticagrelor or Prasugrel (6, 32), that are not depending on a complex hepatic activation as clopidogrel to display their effect, thus suggesting the existence of other mechanisms conditioning platelets activity and thrombotic risk.

Vitamin D has received great attention as a hormonal factor modulating cardiovascular function, as its hydroxylated, active form (1,25-OHD) has been shown to prevent endothelial dysfunction and inflammatory processes (33-34), that represent the pathophysiological basis of atherosclerosis. In fact, vitamin D deficiency has been associated with a higher mortality and an enhanced cardiovascular risk in large scale registries, as in the Multi-Ethnic Study of Atherosclerosis (MESA) (35) and the National Health and Nutrition Examination Survey (NHANES) 2001 to 2006 (36).

However, the interest for the role of vitamin D on thrombotic risk has become even more relevant after the identification of vitamin D receptor (VDR) on platelets surface (37), where studies in vitro and in vivo have suggested that the vitamin D-VDR system could inhibit platelet reactivity (38-39). In fact, we previously documented in a cohort of 503 patients on DAPT that lower vitamin D levels were associated with higher platelet reactivity and impaired effectiveness of the ADP-antagonists Clopidogrel and Ticagrelor (40).

Nevertheless, no study has so far evaluated the impact of vitamin D supplementation on platelet reactivity and contrasting data have been reported on its potential benefits on cardiovascular outcomes (15-17).

Indeed, previous reports have suggested that the variations in the response to the treatment with vitamin D could be dependent on the role of Vitamin D Binding Protein (VDBP), that is the major transporter of the vitamin in the bloodstream, therefore conditioning the pool of vitamin D, the levels of the free active form and its biological effects (41-42).

In fact, in a previous study, Lopez-Farrè et al. (43) reported higher vitamin D binding protein (DBP) levels in ASA resistant subjects and moreover, the addition of VDBP to whole blood of healthy donors significantly reduced the inhibitory effect of aspirin on TxA2 production, thus potentially suggesting that this protein could prevent the binding of vitamin D to its receptor and its antithrombotic effects.

Moreover, common genetic variations of VDBP have been reported to account for more than 80% of the variability of the circulating protein and for 10% of vitamin D levels (19). In particular, the single nucleotide polymorphism rs7041, dependent on a T→G substitution in exon 11, is responsible for the substitution Asp→ Glu, that translates in the allelic variant Gc1S of the protein, associated with more elevated levels of VDBP and an increased affinity for vitamin D.

In the previous ARIC study (44), carriers of the rs7041 substitution, genetically predisposed to low vitamin D levels, showed a slightly increased risk of stroke at a median follow-up of 20 years.

A similar impact of the G allele was documented among more than 500 Lithuanian patients undergoing coronary angiography (45), where the G allele increased the risk of CAD with an odds ratio (OR) equal to 1.45 ($p< 0.02$). In addition, G carriers displayed an increased cardiovascular risk profile according Mateos-Muñoz et al. (46), where this variant was associated with insulin resistance and in the study by Almesri et al. (47), showing an association of G allele with BMI. On the contrary, an opposite association of the T allele with the risk of incident diabetes was observed by Reis et al. (48) among Caucasians in another large scale registry.

However, no study has so far addressed the role of this genetic variant of VDBP on platelet reactivity, and especially among patients on DAPT, that was the aim of the present study. We showed that the genetic status of rs7041 polymorphism of VDBP did not affect platelet reactivity or the occurrence of HRPR on antiplatelet treatment among 400 patients on maintenance DAPT after a recent acute coronary syndrome or elective PCI.

In addition, this polymorphism did not affect vitamin D levels. Nevertheless, we observed a significant interaction between severe vitamin D deficiency and the G allele carriage for rs7041 of VDBP, conditioning more enhanced platelet reactivity and a higher prevalence of poor responders to Clopidogrel/Ticagrelor and especially in homozygotes, suggesting that hypovitaminosis D and its correction could play a more relevant role among these patients, where the reduced bioavailability

of vitamin D induced by its sequestration with VDBP complexes could render even more remarkable the impact of its deficiency.

Therefore, future studies should be drawn to evaluate whether vitamin D supplementation can positively revert platelet hyperreactivity among the carriers of the G allele. In fact, vitamin D supplementation does not affect the genetically determined levels of VDBP, thus suggesting that the administration of exogenous vitamin D could directly impact on its free and biologically active quote (49).

## Limitations

A first limitation can be considered the prospective design of the study, including a consecutive cohort of patients, thus leading to a certain unbalance between the different genetic status of the patients, with almost 80% of the patients carrying the G allele. However, this drawing was made on purpose, allowing to evaluate the weight of genetics in a real-life all-comers population, and to consider separately the homozygotes and heterozygotes for the G allele of VDBP. Moreover, the additive effect of hypovitaminosis D in G/G patients as compared to G/T ones further confirms our data. In addition, we did not collect data at long term follow-up, therefore we could not evaluate whether higher platelet reactivity related to the occurrence of cardiovascular events in our population. However, previous studies have documented the prognostic role of HRPR, as defined by Multiplate aggregometry, on the risk of major cardiovascular events (50-51).

In addition, we did not measure the levels of circulating VDBP, and therefore we could not define the impact of this genetic variant on the total pool of vitamin D-VDBP in our patients. Nevertheless, the role of rs7041 in modulating the levels of VDBP is well established (19) and the identification of a differential effect of hypovitaminosis D on platelet reactivity according to the genetic status certainly support such findings.

## Conclusions

The present study shows that rs741 polymorphism of Vitamin D Binding Protein does not affect platelet reactivity or the rate of HRPR among patients receiving DAPT. However, the carriage of the G allele can condition the impact of hypovitaminosis D on the response to antiplatelet agents, increasing the occurrence of HRPR especially in homozygotes, thus suggesting a more significant role of vitamin D deficiency among these patients.

## References


1. De Luca G, Bellandi F, Huber K, Noc M, Petronio AS, Arntz HR, Maioli M, Gabriel HM, Zorman S, DE Carlo M, Rakowski T, Gyongyosi M, Dudek D. Early glycoprotein IIb-IIIa inhibitors in primary angioplasty-abciximab long-term results (EGYPT-ALT) cooperation: individual patient's data meta-analysis. J Thromb Haemost 2011; 9: 2361-70.
2. Verdoia M, Schaffer A, Barbieri L, Cassetti E, Piccolo R, Galasso G, Marino P, Sinigaglia F, De Luca G. Benefits from new ADP antagonists as compared with clopidogrel in patients with stable angina or acute coronary syndrome undergoing invasive management: a meta-analysis of randomized trials. J Cardiovasc Pharmacol 2014; 63: 339-50.
3. Navarese EP, De Luca G, Castriota F, Kozinski M, Gurbel PA, Gibson CM, Andreotti F, Buffon A, Siller-Matula JM, Sukiennik A, De Servi S, Kubica J. Low-molecular-weight heparins vs. unfractionated heparin in the setting of percutaneous coronary intervention for ST-elevation myocardial infarction: a meta-analysis. J Thromb Haemost. 2011; 9: 1902-15.
4. Tantry US, Bonello L, Aradi D, Price MJ, Jeong YH, Angiolillo DJ, Stone GW, Curzen N, Geisler T, Ten Berg J, Kirtane A, Siller-Matula J, Mahla E, Becker RC, Bhatt DL, Waksman R, Rao SV, Alexopoulos D, Marcucci R, Reny JL, Trenk D, Sibbing D, Gurbel PA.; Working Group on On-Treatment Platelet Reactivity. Consensus and update on the definition of on-treatment platelet reactivity to adenosine diphosphate associated with ischemia and bleeding. J Am Coll Cardiol. 2013;62:2261-2273
5. Nguyen TA, Diodati JG, Pharand C. Resistance to clopidogrel: a review of the evidence. J Am Coll Cardiol 2005;45:1157–1164.
6. Verdoia M, Sartori C, Pergolini P, Nardin M, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Prevalence and predictors of high-on treatment platelet reactivity with ticagrelor in ACS patients undergoing stent implantation. Vascul Pharmacol. 2016 Feb;77:48-53
7. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Alfonso F, Macaya C, Bass TA, Costa MA. Variability in individual responsiveness to clopidogrel: clinical implications, management, and future perspectives. J Am Coll Cardiol 2007; 49:1505–16.
8. Wisman PP, Roest M, Asselbergs FW, de Groot PG, Moll FL, van der Graaf Y, de Borst GJ. Platelet-reactivity tests identify patients at risk of secondary cardiovascular events: a systematic review and meta-analysis. J Thromb Haemost. 2014; 12:736-747
9. Stone GW, Witzenbichler B, Weisz G, Rinaldi MJ, Neumann FJ, Metzger DC, Henry TD, Cox DA, Duffy PL, Mazzaferri E, Gurbel PA, Xu K, Parise H, Kirtane AJ, Brodie BR, Mehran R, Stuckey TD; ADAPT-DES Investigators. Platelet reactivity and clinical outcomes after coronary artery implantation of drug-eluting stents (ADAPT-DES): a prospective multicentre registry study. Lancet. 2013; 382: 614-623
10. Borst O, Muenzer P, Schmid E, Russo A, Walker B, Yang W, LeibrockC, Szteyn K, Schmidt S, Russo A, Walker B, Yang W, Leibrock C, Szteyn K, Schmidt S, Elvers M, Faggio C, Shumilina E, Kuro-o M, Gawaz M, Lang F. 1,25(OH)2 vitamin D3 dependent inhibition of platelet Ca2+ signaling and thrombus formation on klotho-deficient mice. FASEB J. 2014; 28: 2108-2119.
11. Silvain J, Cayla G, Hulot JS, Finzi J, Kerneis M, O'Connor SA, Bellemain-Appaix A, Barthélémy O, Beygui F, Collet JP, Montalescot G. High on-thienopyridine platelet reactivity in elderly coronary patients: the SENIOR-PLATELET study. Eur Heart J. 2012;33:1241-9.
12. Verdoia M, Pergolini P, Rolla R, Nardin M, Schaffer A, Barbieri L, Marino P, Bellomo G, Suryapranata H, De Luca G.Advanced age and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. J Thromb Haemost. 2016 Jan;14(1):57-64
13. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation. Impact of age on long-term outcome after primary angioplasty with bare-metal or drug-eluting stent (from the DESERT cooperation). Am J Cardiol. 2013 Jul 15;112(2):181-6.
14. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation.Impact of diabetes on long-term outcome after primary angioplasty: insights from the DESERT cooperation. Diabetes Care 2013; 36: 1020-5.
15. Wang TJ, Pencina MJ, Booth SL, Jacques PF, Ingelsson E, Lanier K, Benjamin EJ, D'Agostino RB, Wolf M, Vasan RS. Vitamin D deficiency and risk of cardiovascular disease. Circulation. 2008;117(4):503-511
16. Ford JA, MacLennan GS, Avenell A, Bolland M, Grey A, Witham M; RECORD Trial Group. Cardiovascular disease and vitamin D supplementation: trial analysis, systematic review, and meta-analysis. Am J Clin Nutr. 2014;100(3):746-55
17. Elamin MB, Abu Elnour NO, Elamin KB, Fatourechi MM, Alkatib AA, Almandoz JP, Liu H, Lane MA, Mullan RJ, Hazem A, Erwin PJ, Hensrud DD, Murad MH, Montori VM. Vitamin D and cardiovascular outcomes: a systematic review and meta-analysis. J Clin Endocrinol Metab 2011; 96: 1931-42
18. Speeckaert M, Huang G, Delanghe JR, Taes YEC. Biological and clinical aspects of the vitamin D binding protein (Gc-globulin) and its polymorphism. Clin Chim Acta. 2006;372(1-2):33-42.
19. Powe CE, Evans MK, Wenger J, Zonderman AB, Berg AH, Nalls M, Tamez H, Zhang D, Bhan I, Karumanchi SA, Powe NR, Thadhani R. Vitamin D–Binding Protein and Vitamin D Status of Black Americans and White Americans. N Engl J Med. 2013;369(21):1991-2000
20. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P. Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis 2012; 22: 426-433
21. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357: 266-81
22. Verdoia M, Pergolini P, Camaro C, Restifo M, Rolla R, Schaffer A, Di Giovine G, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). PlA(1)/PlA(2) polymorphism does not influence response to Gp IIb-IIIa inhibitors in patients undergoing coronary angioplasty. Blood Coagul Fibrinolysis 2013; 24: 411-8
23. Vidali M, Rolla R, Parrella M, Cassani C, Manzini M, Portalupi MR, Serino R, Prando MD, Bellomo G, Pergolini P. Role of the laboratory in monitoring patients receiving dual antiplatelet therapy. Int J Lab Hematol. 2012; 34: 484-94.
24. Bonello L, Pansieri M, Mancini J, Bonello R, Maillard L, Barnay P, Rossi P, Ait-Mokhtar O, Jouve B, Collet F, Peyre JP, Wittenberg O, de Labriolle A, Camilleri E, Cheneau E, Cabassome E, Dignat-George F, Camoin-Jau L, Paganelli F. High on-treatment platelet reactivity after prasugrel loading dose and cardiovascular events after percutaneous coronary intervention in acute coronary syndromes. J Am Coll Cardiol. 2011 Jul 26;58(5):467-73
25. De Luca G, Dirksen MT, Spaulding C, Kelbæk H, Schalij M, Thuesen L, van der Hoeven B, Vink MA, Kaiser C, Musto C, Chechi T, Spaziani G, Diaz de la Llera LS, Pasceri V, Di Lorenzo E, Violini R, Suryapranata H, Stone GW; DESERT cooperation. Time course, predictors and clinical implications of stent thrombosis following primary angioplasty. Insights from the DESERT cooperation. Thromb Haemost 2013; 110: 826-833.

26. De Luca G, Suryapranata H. Recent Advances in Optimal Adjunctive Antithrombotic Therapy in STEMI Patients Undergoing Primary Angioplasty: An Overview. Curr Vasc Pharmacol. 2015;13(5):594-615.
27. Sibbing D, Braun S, Morath T, Mehilli J, Vogt W, Schömig A, Kastrati A, von Beckerath N. Platelet reactivity after clopidogrel treatment assessed with point-of-care analysis and early drug-eluting stent thrombosis. J Am Coll Cardiol 2009;53:849 –856
28. Tekkeşin Aİ, Kaya A, Çakıllı Y, Türkkan C, Hayıroğlu Mİ, Borklu EB, Kalenderoğlu K, Gümüşdağ A, Yıldırımtürk Ö, Bozbeyoğlu E, Tatlısu MA, Alper AT.The first six-month clinical outcomes and risk factors associated with high on-treatment platelet reactivity of clopidogrel in patients undergoing coronary interventions. Anatol J Cardiol. 2016 Apr 25. [Epub ahead of print]
29. Valenti R, Marcucci R, Capodanno D, De Luca G, Migliorini A, Gori AM, Parodi G, Giusti B, Carrabba N, Paniccia R, Cantini G, Marrani M, Gensini GF, Abbate R, Antoniucci D. Residual platelet reactivity to predict long-term clinical outcomes after clopidogrel loading in patients with acute coronary syndromes: comparison of different cutoff values by light transmission aggregometry from the responsiveness to clopidogrel and stent thrombosis 2-acute coronary syndrome (RECLOSE 2-ACS) study. J Thromb Thrombolysis. 2015;40(1):76-82
30. Ancrenaz V, Daali Y, Fontana P, Besson M, Samer C, Dayer P, Desmeules J. Impact of genetic polymorphisms and drug-drug interactions on clopidogrel and prasugrel response variability. Curr Drug Metab. 2010;11:667-677
31. Alexopoulos D, Xanthopoulou I, Perperis A, Siapika A, Stavrou K, Tsoni E,Davlouros P, Hahalis G. Factors affecting residual platelet aggregation in prasugrel treated patients. Curr Pharm Des. 2013;19(28):5121-6
32. Perl L, Lerman-Shivek H, Rechavia E, Vaduganathan M, Leshem-Lev D, Zemer-Wassercug N, Dadush O, Codner P, Bental T, Battler A, Kornowski R, Lev EI. Response to prasugrel and levels of circulating reticulated platelets in patients with ST-segment elevation myocardial infarction. J Am Coll Cardiol. 2014;63:513-517
33. Sugden JA, Davies JI, Witham MD, Morris AD, Struthers AD. Vitamin D improves endothelial function in patients with type 2 diabetes mellitus and low vitamin D levels. Diab Med 2008; 25: 320–325
34. Kassi E, Adamopoulos C, Basdra EK, Papavassiliou AG. Role of Vitamin D in Atherosclerosis. Circulation. 2013;128(23):2517-2531
35. Bansal N, Zelnick L, Robinson-Cohen C, Hoofnagle AN, Ix JH, Lima JA, Shoben AB, Peralta CA, Siscovick DS, Kestenbaum B, de Boer IH. Serum parathyroid hormone and 25-hydroxyvitamin D concentrations and risk of incident heart failure: the Multi-Ethnic Study of Atherosclerosis. J Am Heart Assoc. 2014;3(6):e001278
36. Zhao G, Ford ES, Li C, Croft JB. Serum 25-hydroxyvitamin D levels and all-cause and cardiovascular disease mortality among US adults with hypertension: the NHANES linked mortality study. J Hypertens. 2012;30(2):284-289.
37. Silvagno F, De Vivo E, Attanasio A, Gallo V, Mazzucco G, Pescarmona G. Mitochondrial localization of vitamin D receptor in human platelets and differentiated megakaryocytes;. PLoS One. 2010 ; 5: e8670.
38. *A*ihara K, Azuma H, Akaike M, Ikeda Y, Yamashita M, Sudo T, Hayashi H, Yamada Y, Endoh F, Fujimura M, Yoshida T, Yamaguchi H, Hashizume S, Kato M, Yoshimura K, Yamamoto Y, Kato S, Matsumoto T. Disruption of nuclear vitamin D receptor gene causes enhanced thrombogenicity in mice. J Biol Chem. 2004; 279:35798-35802
39. Aihara K, Azuma H, Matsumoto T. [Vitamin D-vitamin D receptor system regulates antithrombogenicity in vivo]. Clin Calcium. 2006;16:1173-79
40. Verdoia M, Pergolini P, Rolla R, Sartori C, Nardin M, Schaffer A, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets. 2016;27(6):576-82
41. Delanghe JR, Speeckaert R, Speeckaert MM. Behind the scenes of vitamin D binding protein: more than vitamin D binding. *Best Pract Res Clin Endocrinol Metab*. 2015;29(5):773-786
42. White P, Cooke N. The multifunctional properties and characteristics of vitamin D-binding protein. Trends Endocrinol Metab. 2000;11(8):320-327
43. López-Farré AJ, Mateos-Cáceres PJ, Sacristán D, Azcona L, Bernardo E, de Prada TP, Alonso-Orgaz S, Fernández-Arquero M, Fernández-Ortiz A, Macaya C. Relationship between vitamin D binding protein and aspirin resistance in coronary ischemic patients: a proteomic study. J Proteome Res. 2007;6:2481-7
44. Michos ED, Misialek JR, Selvin E, Folsom AR, Pankow JS, Post WS, Lutsey PL. 25-hydroxyvitamin D levels, vitamin D binding protein gene polymorphisms and incident coronary heart disease among whites and blacks: The ARIC study. Atherosclerosis. 2015;241(1):12-17.
45. Stakisaitis D, Lesauskaitė V, Girdauskaitė M, Janulionis E, Ulys A, Benetis R. Investigation of Vitamin D-Binding Protein Polymorphism Impact on Coronary Artery Disease and Relationship with Longevity: Own Data and a Review. Int J Endocrinol. 2016;2016:8347379
46. Mateos-Muñoz B, García-Martín E, Torrejón MJ, Devesa-Medina MJ, Esguevillas G, Cárdenas MC, Fernández C, Carballo M, Agúndez JA, Ladero JM. GC Gene Polymorphism and Unbound Serum Retinol-Binding Protein 4 Are Related to the Risk of Insulin Resistance in Patients With Chronic Hepatitis C: A Prospective Cross-Sectional Study. Medicine (Baltimore). 2016;95(10):e3019
47. Almesri N, Das NS, Ali ME, Gumaa K, Giha HA. Independent associations of polymorphisms in vitamin D binding protein (GC) and vitamin D receptor (VDR) genes with obesity and plasma 25OHD3 levels demonstrate sex dimorphism. Appl Physiol Nutr Metab. 2016;41(4):345-53
48. Reis JP, Michos ED, Selvin E, Pankow JS, Lutsey PL. Race, vitamin D-binding protein gene polymorphisms, 25-hydroxyvitamin D, and incident diabetes: the Atherosclerosis Risk in Communities (ARIC) Study. *Am J Clin Nutr*. 2015;101(6):1232-1240
49. Medlej-Hashim M, Jounblat R, Hamade A, Ibrahim JN, Rizk F, Azzi G, Abdallah M, Nakib L, Lahoud M, Nabout R. Hypovitaminosis D in a Young Lebanese Population: Effect of GC Gene Polymorphisms on Vitamin D and Vitamin D Binding Protein Levels. Ann Hum Genet. 2015;79(6):394-401
50. Siller-Matula JM, Delle-Karth G, Lang IM, Neunteufl T, Kozinski M, Kubica J, Maurer G, Linkowska K, Grzybowski T, Huber K, Jilma B. Phenotyping vs. genotyping for prediction of clopidogrel efficacy and safety: the PEGASUS-PCI study. J Thromb Haemost. 2012;10:529-42
51. Siller-Matula JM, Gruber C, Francesconi M, Dechant C, Jilma B, Delle-Karth G, Grohs K, Podczeck-Schweighofer A, Christ G. The net clinical benefit of personalized antiplatelet therapy in patients undergoing percutaneous coronary intervention. Clin Sci (Lond). 2015;128:121-30.

# Chapter 7:

# Polymorphism rs 2762939 of CYP24A1 enzyme and coronary artery disease: angiographic results from a large prospective cohort of patients.

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*Submitted*

## ABSTRACT

**Background.** Vitamin D deficiency represents an emerging risk factor for coronary artery disease and cardiovascular outcomes. Recent attention has been focused on the regulation of vitamin D metabolism and homeostasis as modulating the cardiovascular benefits of vitamin D. In particular, the functional impact of the genetic polymorphism rs2762939 of CYP24A1, the hydroxylase-enzyme modulating the inactivation of vitamin D, is still debated. Aim of the present study was to evaluate the relationship between rs2762939 genotype and the prevalence and extent of coronary artery disease in a large cohort of patients undergoing coronary angiography.

**Methods** A consecutive cohort of patients undergoing coronary angiography in a single centre were included. Significant CAD was defined as at least 1 stenosis > 50%, severe CAD for as left main and/or three-vessel disease. CYP24A1 genetic status was assessed by polymerase chain reaction and restriction fragment length polymorphism technique.

**Results** We included in our study 1024 patients, 673 carried the C allele, (wild-type G: 66%; C-allele 34%; Hardy-Weinberg p=0.15). Baseline clinical and demographic features were well balanced between the two groups, but for a lower use of beta-blockers among the C-carriers (p=0.01) and higher levels of C-reactive protein (p=0.05). 30.9% of the patients displayed hypovitaminosis D and 2.1% of patients received vitamin D supplementation, with no difference according to genotype. The prevalence of CAD and severe CAD was not conditioned by CYP24A1 genetic status (78.7% GG vs 81.2% C-carriers; p=0.31; adjusted OR [95% CI]= 0.71 [0.20-2.56], p=0.60 and 29.1% GG vs 29.5% C carriers p=0.95; adjusted OR [95% CI]= 0.87 [0.73-1.04], p=0.13, respectively). However, the prevalence of coronary calcifications was significantly higher among GG homozygotes (p=0.005).

**Conclusion** This study showed in a large cohort of patients undergoing coronary angiography, that the polymorphisms rs2762939 of CYP24A1 is not associated with the prevalence and extent of CAD. However, the C-allele mutation carriage significantly lowers the rate of coronary calcifications.

**Keywords:** *Atherosclerosis; Vitamin D; VDBP; Polymorphisms*

## INTRODUCTION

Recent developments in the field of pharmacological therapy and in the strategies for coronary revascularization have significantly improved the prognosis of patients with coronary artery disease (CAD) (1-3). However, ischemic heart disease still represents the leading cause of mortality worldwide, and preventive measures for contrasting the progression of atherosclerotic disease still fail for a large part in reducing the burden of CAD (4). Several studies have been conducted so far, aiming at the identification of new predictors and early markers of cardiovascular risk (5-7).

Over 10-years of genome-wide association studies have addressed several genetic variants potentially linked to the pathogenesis of CAD, with contrasting results (8).

Vitamin D deficiency, a common condition affecting over half of the general population, has also recently emerged as a major determinant of cardiovascular events and impaired long-term outcomes (9,10). Therefore, the loss of the anti-inflammatory, anti-thrombotic and cardioprotective effects of vitamin D has been pointed as a new potential target for cardiovascular prevention, although not providing, so far, the expected benefits (11). Dysregulation in the metabolism of vitamin D and in its transformation into the hydroxylated, biologically active, isoform (calcitrol or 1,25(OH)2D) have been suggested as potential determinants of this variability of the results (12). In particular, a reduced activity of the CYP24A1 enzyme, responsible for the inactivation of calcitriol, has been associated to its accumulation and nephrocalcinosis (13), whereas an opposite increase has been linked to insufficient effect of vitamin D and cancerogenesis(14). The single nucleotide polymorphism (SNP) rs2762939 of CYP24A1 is dependent from a C→G substitution in intronic position, with a minor allele frequency of 29% in Caucasic population (15), potentially conditioning the protein expression and the activity of CYP24A1. Despite initial promising data linking this variant with the pathogenesis of coronary atherosclerosis, no study has so far directly addressed its role on the prevalence and extent of angiographically defined CAD, that was therefore the aim of the present study.

## METHODS

We included consecutive patients undergoing coronary angiography between October 2012 and June 2016 at the Azienda Ospedaliera-Universitaria, "Maggiore della Carità", Novara, Italy. Informed consent was obtained from all patients before angiography. The protocol was approved by our local Ethical Committee and is in accordance to the Declaration of Helsinki statements. All demographic and clinical data were prospectively collected in a dedicated database. Hypertension was defined as a systolic blood pressure (BP) > 140 mmHg and/or a diastolic BP > 90 mmHg or on-treatment with antihypertensive medications. The diagnosis of diabetes was based on previous history of diabetes treated with or without drugs, fasting glycaemia > 126 mg/dl or glycosylated haemoglobin > 6.5%. Hypercholesterolemia was defined as previous history of hypercholesterolemia, chronic treatment with any cholesterol-lowering agent at admission or fasting total cholesterol > 200 mg/dl.

### *Biochemical measurements*

Blood samples were drawn at admission from patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography for main chemistry and genetic assessment. Glucose, creatinine, uric acid levels, blood cells count and lipid profile were determined by standard methods, as previously described (16). 25-hydroxyvitamin D measurement was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for 25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference (17). 25-hydroxyvitamin D inadequate levels were considered for levels < 10 ng/ml according to the US Endocrine Society guideline (17).

### *CYP24A1 polymorphisms genotyping*

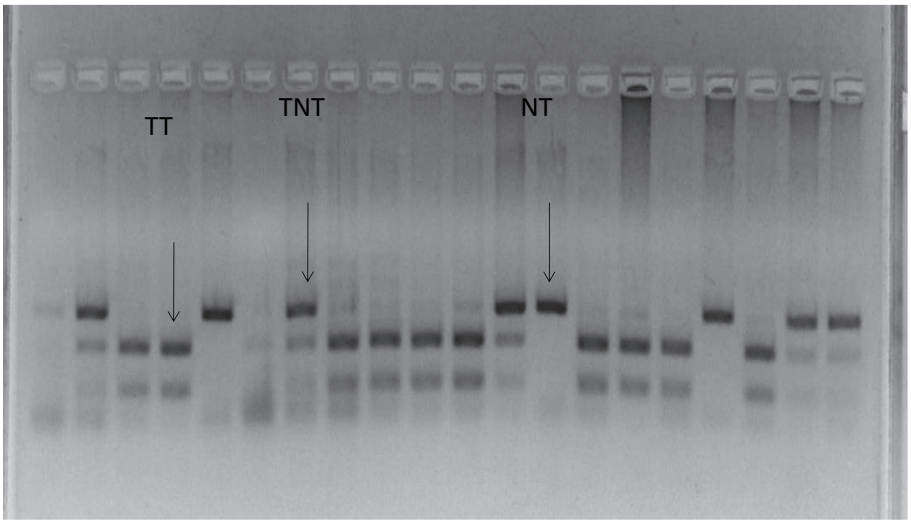
Using a commercially available kit (GenElute™ Blood Genomic DNA, Sigma Aldrich) genomic deoxyribonucleic acid (DNA) was isolated from 200 ml of the peripheral blood samples. The

rs2762939 polymorphisms was studied using combined polymerase chain reaction (PCR) and restriction fragment length polymorphism technique. Following PCR conditions were used: 1.5 µl of genomic DNA were amplified in a mix containing MgCl2 [4 mM], deoxy nucleotides (dNTPs) mix [3%], 5% dimethyl sulfoxide (DMSO), 0.25 µl/100 ml DNA polymerase (GoTaq® Hot Start Polymerase, Promega) and a primers mix [3%], (fwd; 5'-CCAAACGTGCTCATCATCTG-3'' and rev: 5'-ATCAAACACATCCAGTGGAAA-3'), annealing temperature was settled at 54 °C.

The polymorphism presence was evaluated by the pattern of length of restriction fragments after digesting PCR products by Sau96I- Cfr13I (Thermo ScientificBsuRI).

In case of wild-type (G) allele, the enzyme recognised a restriction site resulting in products of 79 and 45 base pairs, while a single fragment of 124 base pairs remained for mutated allele. The digestion product was analysed with electrophoretic run on high resolution agarose 2.15% TAE gel (Figure 1).

Figure 1 Electrophoresis on High Resolution agarose gel after restriction of Polymerase Chain Reaction product with the enzyme Sau96I- Cfr13I (TT= GG homozygotes, TNT= heterozygotes; NT= CC homozygotes)




*Coronary angiography*

Coronary angiography was routinely performed by the Judkins technique, preferring the radial approach, using 6-French right and left heart catheters. Quantitative coronary angiography was performed by two experienced interventional cardiologists (18) who had no knowledge of the

patients' genetic data, by an automatic edge-detection system (Siemens Acom Quantcor QCA, Erlangen, Germany). After the visual inspection of the coronary artery, the frame of optimal clarity was selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, the analysed arterial segment with coronary lesion was defined by moving the cursor from the proximal to the distal part of coronary artery to ensure adequate determination of reference diameter. We have measured minimal luminal diameter, reference diameter, percent diameter stenosis and length of the lesion.

Significant CAD was defined as at least 1 coronary stenosis > 50%. Severe multivessel disease was defined as three-vessel disease and/or left main disease. In case of patients who had previously undergone percutaneous coronary intervention, even though no restenosis was observed, the treated vessel was counted as significantly diseased. In previously bypassed patients, native arteries and grafts were taken into account in the evaluation of extension of artery disease (number of diseased vessels).

*Statistical analysis*

Statistical analysis was performed using SPSS 22.0 statistical package. Continuous data were expressed as mean + SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Patients were grouped according to genetic status of CYP24A1, carriers of the mutated allele either in heterozygosis and homozygosis were considered together. Multivariate logistic regression analysis was performed to evaluate the relationship between genotype and coronary artery disease, after correction for baseline confounding factors that were entered in the model in block. Results were considered statistically significant for a two-tailed $p < 0.05$.

## RESULTS

**Table 1.** Clinical characteristics according to CYP24A1 gene polymorphism rs2762939

| Baseline clinical characteristics | GG (n=531) | C-carriers (n=673) | P value |
|---|---|---|---|
| Age (mean±SD) | 68.1 ± 11.3 | 67.7 ± 11 | 0.58 |
| Male Sex (%) | 69.7 | 70.9 | 0.66 |
| BMI (mean±SD) | 27 ± 4.4 | 27.3 ± 5.2 | 0.28 |
| Hypercholesterolemia(%) | 55.6 | 55.2 | 0.86 |
| Diabetes mellitus (%) | 36.0 | 38.5 | 0.37 |
| Renal failure (%) | 19.2 | 21.8 | 0.37 |
| Active smokers (%) | 21.4 | 24.0 | 0.59 |
| Hypertension (%) | 71.7 | 74.6 | 0.29 |
| History of MI (%) | 26.9 | 23.1 | 0.14 |
| Previous PCI (%) | 36.7 | 30.8 | 0.36 |
| Previous CABG (%) | 10.7 | 9.7 | 0.57 |
| **Indication to angiography** | | | 0.93 |
| Stable angina/ silent ischemia (%) | 28.5 | 25.5 | |
| STEMI/ACS (%) | 52.1 | 57.8 | |
| Cardiomyopathy/ valvular | 19.4 | 16.7 | |
| **Concomitant medications** | | | |
| ACE inhibitors (%) | 37.1 | 36.4 | 0.81 |
| ARB (%) | 24.0 | 26.8 | 0.29 |
| Beta blockers (%) | 55.2 | 52.6 | 0.38 |
| Nitrates (%) | 33.2 | 33.4 | 0.99 |
| Statins (%) | 59.0 | 51.9 | 0.01 |
| ASA (%) | 64.3 | 61.1 | 0.25 |
| Clopidogrel (%) | 19.9 | 18.5 | 0.55 |
| Calcium antagonists (%) | 23.7 | 20.1 | 0.14 |
| Diuretics (%) | 32.6 | 31.9 | 0.80 |
| **Biochemistry parameters** | | | |
| Platelets (10^6/ml; mean± SD) | 225.2 ± 63.4 | 226.6 ± 70.9 | 0.73 |
| Haemoglobin (g/dl) | 13.4 ± 1.7 | 13.4 ± 1.7 | 0.68 |
| WBC (10^3/ml;mean± SD) | 8.07 ± 2.9 | 8.2 ± 2.9 | 0.43 |
| HDL cholesterol (mg/dL) | 43.4 ± 13.2 | 42.7 ± 13.2 | 0.38 |
| LDL cholesterol (mg/dl) | 91.7 ± 34.8 | 92.4 ± 35.5 | 0.71 |
| Glycaemia (mg/dL) | 121.6 ± 45.7 | 124.2 ± 53.4 | 0.37 |
| Glycosylated haemoglobin (%) | 6.3 ± 2.9 | 6.5 ± 2.9 | 0.51 |
| Creatinine (mg/dL) | 1 ± 0.6 | 1 ± 0.5 | 0.88 |
| C reactive protein (mg/dL) | 0.9 ± 2.02 | 1.2 ± 2.6 | 0.05 |
| Uric acid (mg/dl) | 6.2 ± 3.7 | 6.4 ± 4.3 | 0.42 |

*CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers;*

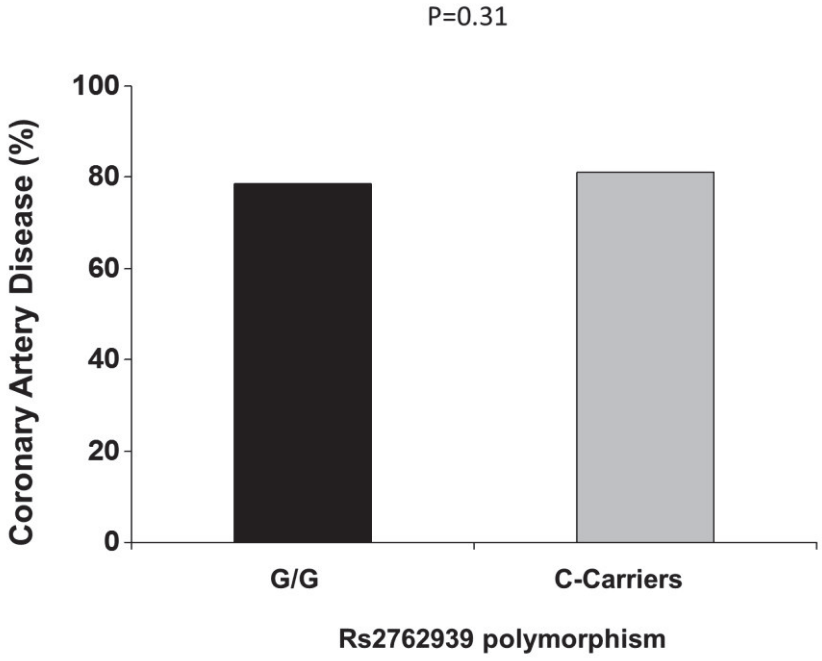
We included in our study 1024 patients, of whom 673 carried the C allele, 153(12.7%) in homozygosis. The frequency of the two-alleles, then, was: wild-type G: 66%; C-allele 34%; that respected the Hardy-Weinberg equilibrium (p=0.15).

Baseline clinical and demographic features are displayed in Table 1 and were well balanced between the two groups, but for a lower use of beta-blockers among the C-carriers (p=0.01) and higher levels of C-reactive protein (p=0.05).

30.9% of the patients displayed hypovitaminosis D (< 10 ng/ml: 30% GG vs 31.5% C-carriers, p=0.64) and 21 (2.1%) of patients received vitamin D supplementation, with no difference according to genotype.

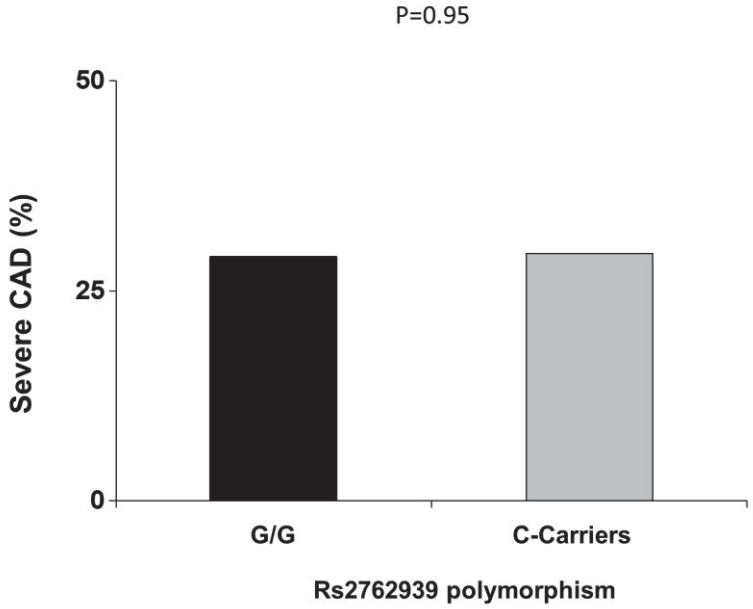
The prevalence of CAD was not conditioned by CYP24A1 genetic status (78.7% GG vs 81.2% C-carriers; p=0.31; as in Figure 2).

Figure 2 Polymorphism rs2762939 and prevalence of coronary artery disease (CAD).




Similar results were obtained for severe CAD, as displayed in Figure 3 (29.1% GG vs 29.5% C carriers p=0.95). Results were confirmed at multivariate analysis, after correction for baseline differences (CAD: adjusted OR [95% CI]= 0.71 [0.20-2.56], p=0.60 and; severe CAD: adjusted OR [95% CI]= 0.87 [0.73-1.04], p=0.13, respectively).

Figure 3. Polymorphism rs2762939 and prevalence of severe coronary artery disease (CAD).




However, as displayed in Table 2, the prevalence of coronary calcifications was significantly higher among GG homozygotes (p=0.005).

Table 2. Angiographic characteristics according to CYP24A1 gene polymorphism rs2762939

| Angiographic features | GG (n=940) | C-carriers (n=1168) | P value |
|---|---|---|---|
| Left main disease (%)§ | 8.1 | 8 | 0.99 |
| LAD (%)§ | 54.7 | 58.8 | 0.174 |
| CX (%)§ | 48.2 | 44.3 | 0.20 |
| RCA (%)§ | 5.1 | 7.0 | 0.21 |
| Type C Lesion (%) | 44.0 | 48.4 | 0.14 |
| Lesion length (mm) | 22.2 ± 13.9 | 22.2 ± 13.9 | 0.92 |
| Percent stenosis (%) | 2.9 ± 0.5 | 2.9 ± 0.6 | 0.23 |
| Reference Diameter (mm) | 85.9 ± 13 | 86.2 ± 13 | 0.62 |
| Bifurcations (%) | 20.8 | 22.0 | 0.52 |
| Calcifications (%) | 18.5 | 14.0 | 0.005 |
| Chronic occlusion (%) | 7.0 | 6.7 | 0.69 |
| Restenosis (%) | 7.2 | 6.7 | 0.25 |
| Thrombus (%) | 5.1 | 5.5 | 0.77 |
| TIMI Flow | | | 0.14 |
| 3 | 76.5 | 73.9 | |
| 2 | 1.9 | 4.7 | |
| 1 | 2.9 | 3.2 | |
| 0 | 18.7 | 18.2 | |

## DISCUSSION

This is the first study conducted so far to evaluate the relationship between CYP24A1 polymorphisms and the prevalence and extent of angiographically defined CAD in a large cohort of consecutive patients. The main finding is that this polymorphism is not associated with the prevalence and extent of coronary disease. However, the wild-type G allele, in homozygosis, significantly increases the prevalence of coronary calcifications.

Accurate assessment of cardiovascular (CV) risk is essential for clinical decision-making, allowing the early identification and more aggressive treatment of those higher-risk subjects (19). Nevertheless, models and scores based on the well-established risk factors still leave a large gap in the prediction of cardiovascular ischemic events (20) and moreover, the addition of several biomarkers for the adequate stratification of the patients has offered so far inconclusive results (21).

Vitamin D deficiency represents not only an emerging social problem, affecting over 1 billion of subjects worldwide (22), but also a relevant prognostic condition for the cardiovascular outcomes, being potentially involved in the pathogenesis of atherothrombosis (23).

In the NHANES III study subjects with vitamin D levels < 20 ng/mL showed an increased occurrence of angina, myocardial infarction and heart failure (24), in the ARIC (Atherosclerosis Risk in Communities) (25), subjects in the lowest vitamin D quintile displayed an increased risk of stroke and also a recent meta-analysis including 8 cohorts of European/US patients confirmed an increased cardiovascular mortality for lower vitamin D levels (26). In addition, similar results have been achieved in more recent cohort studies documenting an association between vitamin D deficiency and the angiographical prevalence of CAD or platelet reactivity, especially among subsets of patients at higher cardiovascular risk (27-29).

Nevertheless, contrasting data have been reported so far on the potential benefits of vitamin D supplementation on cardiovascular outcomes, both in consequence of inadequate design of the studies and in presence of several factors modulating vitamin D levels and action (30,31).

In fact, calcitriol preserves vascular system functions through several mechanisms, including the inhibition of intima-media thickening, enhancement of endothelial nitric oxide synthase, inhibition of macrophages activation and conversion into foam cells; prevention of calcium deposition in vessels, endothelial protection against advanced glycation end products (AGEs) and anti-thrombotic activity, effects that are mainly mediated by the interaction of the activated / hydroxylated 1,25(OH)2D with the vitamin D receptor (32).

Therefore, genetic variants of vitamin D receptor and transporter and in the enzymes regulating the metabolism, hydroxylation and inactivation, of vitamin D have been suggested to interfere with the response to vitamin D.

In particular, modifications in the function of the CYP24A1 hydroxylase, responsible for the 24-hydroxylation and inactivation of calcitriol, have been proposed as modulating its levels and effect (33).

The rs2762939 is a single nucleotide polymorphism (SNP) dependent from a C→G substitution in intronic position in the CYP24A1 enzyme. The C-allele is reported in 29% of the Caucasic population and, despite representing a synonymous mutation, it has been suggested to condition the protein synthesis, the expression of the enzyme and therefore its function. Previous reports, in fact, have linked this genetic variant with increased levels of vitamin D and 1,25(OH)2D (34,35).

However only one study has so far addressed the cardiovascular effects of rs2762939 in cardiovascular disease, with promising results. In fact, Shen et al. (15) documented in 3 different cohorts of patients an association between this genetic variant and coronary artery calcifications (CAC quantity), as measured by electron beam computed tomography.

Our study represents the first report addressing the role of this CYP24A1 polymorphism on coronary artery disease, evaluated at angiography.

We documented in a large population of 1024 patients that the wild-type homozygotes display an increased rate of coronary calcifications, although the overall prevalence of CAD and severe CAD was not affected by the genetic status.

Indeed, an opposite association of rs2762939 was documented for the levels of C-reactive protein, a marker that has been previously associated with cardiovascular risk and the progression of atherosclerosis. However, several other conditions can be responsible for non-specific raise in the levels of CRP, such as acute presentation or diabetic status, that were numerically more common in the ”C-allele” carriers, therefore providing potential explanation for this observation.

However, our results support the previous data by Shen et al. and the reports derived in vitro, suggesting that higher calcitriol levels could prevent coronary calcification by reducing the accumulation of the lipid plaque, that constitutes the basis for calcium deposition and lowering the inflammatory response, preventing macrophages activation and vascular smooth-muscle cells activation (36).

Therefore, present findings pave the way to further, larger studies, that should preferentially focus on the levels of calcitriol and the homeostasis of vitamin D activation, rather than on 25(OH)D. In fact, calcitriol, but not 25(OH)D, has been shown to improve endothelial function and survival in previous small studies (37,38).

*Study limitations*

Limitations of the present study include those inherent to any prospective but observational study. In particular, the differential distribution of cardiovascular risk factors, although non-statistically significant, could have partially represented a confounder, also accounting for the difference in the levels of C-reactive protein observed with genotype in our study. In addition, the low prevalence of

C-allele homozygotes did not allow us to consider them separately from the heterozygotes, therefore reducing the power of our analysis.

Moreover, we did not collect the data on 1,25(OH)2D in our patients, therefore we cannot provide a clear correlation between calcitrol levels, genetic status and the degree of coronary calcifications.

In addition, the evaluation of the extent of CAD and of coronary calcium was performed at angiography, and we did not use additional methods of imaging as intracoronary ultrasound (IVUS) or optical coherence tomography, for the assessment of coronary calcification.

Finally, we did not collect follow-up data, especially in patients undergoing coronary angioplasty, and thus cannot exclude an impact of this polymorphism of the progression of CAD, occurrence of acute MI and risk of adverse events after PCI.

## CONCLUSION

This study showed in a large cohort of patients undergoing coronary angiography, that the polymorphisms rs2762939 of CYP24A1 is not associated with the prevalence and extent of CAD. However, the C-allele mutation carriage significantly lowers the rate of coronary calcifications.

# BIBLIOGRAPHY


1. Nardin M, Verdoia M, Barbieri L, Schaffer A, Suryapranata H, De Luca G. Radial vs Femoral Approach in Acute Coronary Syndromes: A Meta- Analysis of Randomized Trials. Curr Vasc Pharmacol. 2017;16(1):79-92
2. De Luca G, Di Lorenzo E. Everolimus-eluting stent versus Sirolimus-eluting stent for prognostic significance. Int J Cardiol. 2016 ;202:940
3. De Luca G, Van't Hof AW, Gibson CM, Cutlip D, Zeymer U, Noc M, Maioli M, Zorman S, Gabriel HM, Emre A, Rakowski T, Gyongyosi M, Huber K, Bellandi F, Dudek D; EGYPT cooperation. Impact of time from symptom onset to drug administration on outcome in patients undergoing glycoprotein IIb-IIIa facilitated primary angioplasty (from the EGYPT cooperation). Am J Cardiol. 2015;115(6):711-5
4. Stone NJ. Reducing residual risk in secondary prevention of cardiovascular disease. Circulation. 2012;125(16):1958-60
5. Verdoia M, Nardin M, Rolla R, Marino P, Bellomo G, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Immature platelet fraction and the extent of coronary artery disease: A single centre study. Atherosclerosis. 2017;260:110-115
6. Verdoia M, Barbieri L, Di Giovine G, Marino P, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Neutrophil to Lymphocyte Ratio and the Extent of Coronary Artery Disease: Results From a Large Cohort Study. Angiology. 2016;67(1):75-82
7. Schaffer A, Verdoia M, Cassetti E, Marino P, Suryapranata H, De Luca G; Novara Atherosclerosis Study Group (NAS). Relationship between homocysteine and coronary artery disease. Results from a large prospective cohort study. Thromb Res. 2014;134(2):288-93
8. Erdmann J, Kessler T, Munoz Venegas L, Schunkert H. A decade of genome-wide association studies for coronary artery disease: The challenges ahead. Cardiovasc Res. 2018 Mar 30 [Epub ahead of print]
9. Norman PE, Powell JT. Vitamin D and Cardiovascular Disease. Circulation Research. 2014;114:379-393.
10. Pilz S, Trummer C, Pandis M, Schwetz V, Aberer F, Grübler M, Verheyen N, Tomaschitz A, März W. Vitamin D: Current Guidelines and Future Outlook. Anticancer Res. 2018;38(2):1145-1151
11.Elamin MB., Abu Elnour NO., Elamin KB., Fatourechi MM., Alkatib AA., Almandoz JP., Liu H., Lane MA., Mullan RJ., Hazem A., Erwin PJ., Hensrud DD., Murad MH., Montori VM. Vitamin D and cardiovascular outcomes: a systematic review and meta-analysis. Journal Clinical of Endocrinology & Metabolism 2011;96:1931-42
12. Mozos I, Marginean O. Links between Vitamin D Deficiency and Cardiovascular Diseases. Biomed Res Int. 2015;2015:109275
13. Schlingmann K.P., Kaufmann M., Weber S.. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. New England Journal Medicine. 2011;365:410-21
14. Ping Wang, Hemei Zhang, Zengli Zhang, Liqiang Qin, and Bingyan Li Association of the CYP24A1-rs2296241 polymorphism of the vitamin D catabolism enzyme with hormone-related cancer risk: a meta-analysis. 2015;8: 1175-1183
15. Shen H, Bielak LF, Ferguson JF, Streeten EA, Yerges-Armstrong LM, Liu J, Post W, O'Connell JR, Hixson JE, Kardia SL, Sun YV, Jhun MA, Wang X, Mehta NN, Li M, Koller DL, Hakonarson H, Keating BJ, Rader DJ, Shuldiner AR, Peyser PA, Reilly MP, Mitchell BD. Association of the vitamin D metabolism gene CYP24A1 with coronary artery calcification. Arterioscler Thromb Vasc Biol. 2010 ;30(12):2648-54
16. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P; Novara Atherosclerosis Study Group (NAS). Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012;22:426-33.
17. Holick MF. Vitamin D Deficiency. N Engl J Med. 2007;357:266-281.
18. Daffara V, Verdoia M, Rolla R, Nardin M, Marino P, Bellomo G, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of polymorphism rs7041 and rs4588 of Vitamin D Binding Protein on the extent of coronary artery disease. Nutr Metab Cardiovasc Dis. 2017 Sep;27(9):775-783
19. Conroy RM, Pyörälä K, Fitzgerald AP, Sans S, Menotti A, De Backer G, De Bacquer D, Ducimetière P, Jousilahti P, Keil U, Njølstad I, Oganov RG, Thomsen T, Tunstall-Pedoe H, Tverdal A, Wedel H, Whincup P, Wilhelmsen L, Graham IM; SCORE project group. Estimation of ten-year risk of fatal cardiovascular disease in Europe: the SCORE project. Eur Heart J. 2003;24(11):987-1003
20. Siontis GC, Tzoulaki I, Siontis KC, Ioannidis JP. Comparisons of established risk prediction models for cardiovascular disease: systematic review. BMJ. 2012 ;344:e3318
21. Vlachopoulos C, Xaplanteris P, Aboyans V, et al. The role of vascular biomarkers for primary and secondary prevention. A position paper from the European Society of Cardiology Working Group on peripheral circulation. Endorsed by the Association for Research into Arterial Structure and Physiology Artery. Atherosclerosis. 2015;241:507-532.
22. Naeem Z. Vitamin D Deficiency- An Ignored Epidemic. International Journal of Health Sciences. 2010;4(1):V-VI.
23. Kunadian V, Ford GA, Bavamia B et al. Vitamin D deficiency and coronary artery disease: A review of the evidence. Am Heart J 2014;167:283-91
24. Kendrick J, Targher G, Smits G, Chonchol M. 25-Hydroxyvitamin D deficiency is independently associated with cardiovascular disease in the Third National Health and Nutrition Examination Survey. Atherosclerosis. 2009;205:255-260.
25. Schneider ALC, Lutsey PL., Selvin E. Vitamin D, vitamin D binding protein gene polymorphisms, race and risk of incident stroke: The Atherosclerosis Risk in Communities (ARIC) study. European Journal Neurology. 2015;22:1220-1227.
26. Schöttker B., Jorde R., Peasey A., Thorand B., Jansen EH., Groot LD., Streppel M., Gardiner J., Ordóñez-Mena JM., Perna L., Wilsgaard T., Rathmann W., Feskens E, Kampman E, Siganos G, Njølstad I, Mathiesen EB, Kubínová R, Pająk A., Topor-Madry R., Tamosiunas A., Hughes M., Kee F., Bobak M., Trichopoulou A., Boffetta P., Brenner H. Consortium on Health and Ageing: Network of Cohorts in Europe and the United States.Vitamin D and mortality: meta-analysis of individual participant data from a large consortium of cohort studies from Europe and the United States. British Medicine Journal. 2014;348:3656.
27. Nardin M, Verdoia M, Schaffer A, Barbieri L, Marino P, De Luca G; Novara Atherosclerosis Study Group (NAS). Vitamin D status, diabetes mellitus and coronary artery disease in patients undergoing coronary angiography. Atherosclerosis. 2016;250:114-21.
28. Verdoia M, Pergolini P, Rolla R, Sartori C, Nardin M, Schaffer A, Barbieri L, Daffara V, Marino P, Bellomo G, Suryapranata H, Luca GD; Novara Atherosclerosis Study Group (NAS). Vitamin D levels and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor. Platelets. 2016;27:576-82.
29. Verdoia M, Schaffer A, Sartori C, Barbieri L, Cassetti E, Marino P, Galasso G, De Luca G. Vitamin D deficiency is independently associated with the extent of coronary artery disease. Eur J Clin Invest. 2014;44(7):634-42
30. Avenell A, MacLennan GS, Jenkinson DJ, McPherson GC, McDonald AM, Pant PR, Grant AM, Campbell MK, Anderson FH, Cooper C, Francis RM, Gillespie WJ, Robinson CM, Torgerson DJ, Wallace WA; RECORD Trial Group.. Long-term follow-up for mortality and cancer in a randomized placebo-controlled trial of vitamin D(3) and/or calcium (RECORD trial). J Clin Endocrinol Metab. 2012;97:614-22.
31. Scragg R, Stewart AW, Waayer D, Lawes CMM, Toop L, Sluyter J, Murphy J, Khaw KT, Camargo CA Jr. Effect of Monthly High-Dose Vitamin D Supplementation on Cardiovascular Disease in the Vitamin D Assessment Study : A Randomized Clinical Trial. JAMA Cardiol. 2017 ;2(6):608-616
32. Caccamo D, Ricca S, Currò M, Ientile R. Health Risks of Hypovitaminosis D: A Review of New Molecular Insights. Int J Mol Sci. 2018;19(3)

33. Nigwekar SU, Thadhani R. Vitamin D receptor activation: cardiovascular and renal implications. Kidney Int Suppl (2011). 2013;3(5):427-430
34. Hibler EA, Klimentidis YC, Jurutka PW, Kohler LN, Lance P, Roe DJ, Thompson PA, Jacobs ET. CYP24A1 and CYP27B1 Polymorphisms, Concentrations of Vitamin D Metabolites, and Odds of Colorectal Adenoma Recurrence. Nutr Cancer. 2015;67(7):1131-41
35.Barry E.L. Genetic variants in CYP2R1, CYP24A1, and VDR modify the efficacy of vitamin D3 supplementation for increasing serum 25-hydroxyvitamin D levels in a randomized controlled trial. The Journal of Clinical Endocrinology & Metabolism. 2014;99:E2133-E2137
36. Noonan W, Koch K, Nakane M, Ma J, Dixon D, Bolin A, Reinhart G. Differential effects of vitamin D receptor activators on aortic calcification and pulse wave velocity in uraemic rats. Nephrol Dial Transplant. 2008;23(12):3824-30
37. Shoji T, Marubayashi S, Shigematsu T, Iseki K, Tsubakihara Y; Committee of Renal Data Registry; Japanese Society for Dialysis Therapy. Use of vitamin D receptor activator, incident cardiovascular disease and death in a cohort of hemodialysis patients. Ther Apher Dial. 2015;19(3):235-44
38. Trummer C, Schwetz V, Pandis M, Grübler MR, Verheyen N, Gaksch M, Zittermann A, März W, Aberer F, Lang A, Friedl C, Tomaschitz A, Obermayer-Pietsch B, Pieber TR, Pilz S, Treiber G. Effects of Vitamin D Supplementation on IGF-1 and Calcitriol: A Randomized-Controlled Trial. Nutrients. 2017;9(6)

# Part 4

## *Calcitriol in Acute Coronary Syndromes*

# Chapter 8:

# Determinants of vitamin D activation in patients with acute coronary syndromes and its correlation with inflammatory markers.

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Submitted

ABSTRACT

**Background.** Vitamin D deficiency is a pandemic affecting over 1 billion of subjects worldwide. Calcitriol (1,25(OH)2D) represents the perpetrator of the several systemic effects of vitamin D, including the anti-inflammatory, antithrombotic and anti-atherosclerotic action potentially preventing acute cardiovascular ischemic events. Variability in the transformation of vitamin D into 1,25(OH)2D has been suggested to modulate its cardioprotective benefits, however, the determinants of the levels of calcitriol and their impact on the cardiovascular risk have been seldom addressed and were therefore the aim of the present study.

**Methods** A consecutive cohort of patients undergoing coronary angiography for acute coronary syndrome (ACS) were included. The levels of 25 and 1,25(OH)2 D were assessed at admission by chemiluminescence immunoassay kit LIAISON® Vitamin D assay (DiasorinInc) and LIAISON® XL. Hypovitaminosis D was defined for 25(OH)D < 10 ng/ml, whereas 1,25(OH)2D deficiency for levels < 19.9 pg/ml.

**Results** We included in our study 144 patients, divided according to median values of 1,25(OH)2D (< or ≥ 41.2 pg/ml). Lower calcitriol was associated to diabetes (p=0.05), renal failure (p=0.02), hypertension (p=0.001), use of calcium-antagonists (p=0.001), diuretics (p=0.007), higher creatinine (p=0.02), uric acid (p=0.04), and worse glycemic and lipid profile.

37 patients (25.7%) had hypovitaminosis D, whereas 11 (7.9%) displayed calcitriol < 19.9 pg/ml (16.2% among patients with hypovitaminosis D and 4.9% with normal levels, p=0.06).

The independent predictors of 1,25(OH)2D deficiency were hypertension (OR[95%CI]=3.72[1.21-11.4], p=0.002), use of diuretics (OR[95%CI]=2.5[1.01-6.24], p=0.047), while negative predictor was the level of vitamin D (OR[95%CI]=0.930.89-0.98], p=0.008).

Calcitriol levels, in fact, directly related with the levels of vitamin D (r=0.27, p=0.01), whereas an inverse linear relationship was observed with major inflammatory and metabolic markers of cardiovascular risk, (C-reactive protein: r=-0.27, p=0.002; uric acid: r=-0.24, p=0.03; homocysteine: r=-0.27, p=0.002; fibrinogen: r=-0.28, p=0.002), but for white blood cell count (r=-0.05, p=0.56) and Lp-PLA2 (r=-0.05, p=0.56).

**Conclusion** The present study shows that among ACS patients, hypovitaminosis D is frequent, whereas being associated with sufficient levels of calcitriol in the majority of cases. We identified hypertension, use of diuretics and lower vitamin D as independent predictors of 1,25(OH)2D deficiency. Moreover, we documented a significant inverse relationship of calcitriol with C-reactive protein, uric acid, homocysteine, and fibrinogen, inflammatory and metabolic biomarkers strictly linked to cardiovascular risk

**Keywords:**Vitamin D; *calcitriol, Inflammation, Biomarkers, Acute Coronary Syndrome*

## INTRODUCTION

Vitamin D deficiency represents a rising social problem, affecting a huge part of the population of every age and ethnicity and even over 50% of healthy subjects (1).

In the last years, evidence has emerged for several non-calcium dependent hormonal effects of vitamin D, linking its deficiency to increased mortality and to various chronic pathological conditions, as cancer, neurological disorders, as well as autoimmune and inflammatory diseases, hypertension, diabetes and atherothrombotic cardiovascular events (2,3). However, vitamin D supplementation for the prevention of cardiovascular risk has not provided so far clear evidence of benefit, both due to the lack of well conducted dedicated trials and for the potential interference of several conditions that could modulate the response to the treatment (4,5). In particular, the systemic effects of vitamin D are mediated by the interaction of calcitriol (1,25(OH)2D) with vitamin D receptor (VDR), therefore genetic variants of the VDR, as much as factors regulating the transformation of vitamin D into its active hydroxylated hormone, calcitriol, have been addressed for defining the interindividual variability of the effect of vitamin D (6,7). Despite the evaluation of the precursor (25(OH)D) is generally preferred above the assessment of calcitriol levels, for its longer plasma half-life, previous studies have instead associated the levels of 1,25(OH)2D to cardiovascular disease and worse prognosis, being more subject to transient variations in the context of acute events, as potentially in the settings of an acute coronary syndrome (ACS) (8,9). In addition, although the efficiency of calcitriol hydroxylation has been strictly related to renal function (10), representing the primary site for its transformation, extra-renal production of 1,25(OH)2D has also been documented (11), based on the identification of the responsible 1α-hydroxylase enzyme in several tissues and districts, including the cardiovascular system, but whose exact role is largely undefined, especially in patients without severe renal disease. Aim of the present study was then to evaluate the determinants of calcitriol levels in a high-cardiovascular risk population of ACS patients and to evaluate their relationship with inflammatory biomarkers.

## METHODS

We included consecutive patients undergoing coronary angiography for an acute coronary syndrome between November 2016 and January 2018 at the Azienda Ospedaliera-Universitaria, "Maggiore della Carità", Novara, Italy. Informed consent was obtained from all patients before angiography.

ACS was defined for Unstable Angina, NSTEMI or ST-segment elevation myocardial infarction, and therefore as patients were defined by the presence of chest pain at rest lasting > 20 minutes, with or without cardiac biomarkers elevation > ULN (respectively 0,04 µg/l for Troponin I and 5,00 µg/l for CK-MB) or electrocardiographic modifications (either ST-segment depression or elevation $\geq$2 mm in at least 2 contiguous leads or new LBB onset or T waves change).

The protocol was approved by our local Ethical Committee and is in accordance to the Declaration of Helsinki statements. All demographic and clinical data were prospectively collected in a dedicated database. Hypertension was defined as a systolic blood pressure (BP) > 140 mmHg and/or a diastolic BP > 90 mmHg or on-treatment with antihypertensive medications. The diagnosis of diabetes was based on previous history of diabetes treated with or without drugs, fasting glycaemia > 126 mg/dl or glycosylated haemoglobin > 6.5%. Hypercholesterolemia was defined as previous history of hypercholesterolemia, chronic treatment with any cholesterol-lowering agent at admission or fasting total cholesterol > 200 mg/dl.

### *Biochemical measurement*

Blood samples were drawn at admission from patients undergoing elective (following a fasting period of 12 h) or urgent coronary angiography. Glucose, creatinine, uric acid levels, blood cells count and lipid profile were determined by standard methods, as previously described (12). 25-hydroxyvitamin D and 1,25(OH)2D measurement was performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc) and LIAISON® XL respectively. The normal range for 25-OH D3 levels in our laboratory is from 30 to 100 ng/ml, according to literature reference (13). 25-hydroxyvitamin D

inadequate levels were considered for levels < 10 ng/ml according to the US Endocrine Society guideline (13). Calcitriol deficiency was defined for levels < 19.9 pg/ml according to the manufacturer.

Lipoprotein-associated Phospholipase A2 (Lp-PLA2) was analyzed on ADVIA® 1800 Clinical Chemistry Analyzer (Siemens Healthcare Diagnostics). For the quantitative determination of Lp-PLA2 activity, the Diazyme Lp-PLA2 Activity Assay was utilized (Diazyme Laboratories, CA 92064, USA). Diazyme's Lp- PLA2 Activity Assay is an enzymatic assay. Lp-PLA2 in a sample hydrolyzes the acetyl group at the sn-2 position of phospholipids, 1- myristoyl-2-(4-nitrophenylsuccinyl)-sn-glycero-3-phosphocholine (MNP) to generate 4-nitrophenyl group, a colorful product, which can be monitored spectrophotometrically at 405 nm. The activity of Lp-PLA2 in the sample is proportional to the absorbance increase. The instrument calculates the Lp- PLA2 activity of a sample by using the Diazyme Lp-PLA2 Activity Calibrator Set (REF DZ331A-CAL) to generate a calibration curve (14).

### *Statistical analysis*

Statistical analysis was performed using SPSS 22.0 statistical package. Continuous data were expressed as mean + SD and categorical data as percentage. Analysis of variance and the chi-square test were used for continuous and categorical variables, respectively. Patients were grouped according to median values of 1,25(OH)2D. Forward conditional logistic regression analysis was performed to evaluate the independent predictors of calcitriol deficiency. Linear regression analysis was applied for evaluating the relationship of calcitriol with other continuous laboratory parameters. Results were considered statistically significant for a two-tailed $p < 0.05$.

## RESULTS

We included in our study 144 patients, divided according to median values of 1,25(OH)2D (< or ≥ 41.2 pg/ml).

Major clinical and demographic features of the included population are displayed in Table 1.

**Table 1.** Clinical characteristics according to median values of 1,25(OH)2D

| Baseline clinical characteristics | < 41.2 pg/ml (n=72) | ≥41.2 pg/ml (n=72) | P value |
|---|---|---|---|
| Age (mean±SD) | 69.6±12.6 | 67.4±11.3 | 0.28 |
| Weight (Kg±SD) | 78±17 | 74±14.3 | 0.14 |
| Male Sex (%) | 83.3 | 69.4 | 0.08 |
| BMI (mean±SD) | 27.3±5.1 | 26.5±4.6 | 0.32 |
| Hypercholesterolemia(%) | 50 | 63.9 | 0.13 |
| Diabetes mellitus (%) | 33.3 | 18.1 | 0.05 |
| Renal failure (%) | 26.4 | 9.7 | 0.02 |
| Active smokers (%) | 23.6 | 2.2 | 0.63 |
| Hypertension (%) | 84.7 | 59.7 | 0.001 |
| History of MI (%) | 22.2 | 23.6 | 0.99 |
| Previous PCI (%) | 26.8 | 26.4 | 0.99 |
| Previous CABG (%) | 8.3 | 9.7 | 0.99 |
| **ACS type (%)** | | | 0.14 |
| NSTEMI (%) | 65.3 | 77.8 | |
| STEMI (%) | 34.7 | 22.2 | |
| Left Main-Trivessel CAD (%) | 52.5 | 44.6 | 0.46 |
| **Concomitant medications** | | | |
| ACE inhibitors (%) | 27.1 | 25 | 0.85 |
| ARB (%) | 30 | 19.4 | 0.17 |
| Beta blockers (%) | 52.1 | 43.1 | 0.32 |
| Nitrates (%) | 19.7 | 13.9 | 0.38 |
| Statins (%) | 46.5 | 40.8 | 0.61 |
| ASA (%) | 54.9 | 45.8 | 0.32 |
| Clopidogrel (%) | 11.3 | 8.3 | 0.59 |
| Calcium antagonists (%) | 36.6 | 12.5 | 0.001 |
| Diuretics (%) | 42.3 | 20.8 | 0.007 |
| Vitamin D (%) | 3.2 | 12.5 | 0.08 |
| **Biochemistry parameters** | | | |
| Platelets (10^6/ml; mean± SD) | 218.9±68.3 | 237.1±37.5 | 0.11 |
| Haemoglobin (g/dl) | 13±2.2 | 13.5±1.6 | 0.4 |
| WBC (10^3/ml;mean± SD) | 9.8±4 | 9±2.6 | 0.15 |
| HDL cholesterol (mg/dL) | 41.7±11.5 | 47.6±14.9 | 0.009 |
| LDL cholesterol (mg/dl) | 98.3±34 | 112.3±40 | 0.03 |
| Glycaemia (mg/dL) | 135.3±61 | 114.2±29.8 | 0.01 |
| HbA1c (%) | 6.2±1.2 | 5.8±0.8 | 0.04 |
| 25(OH)D (ng/ml) | 16.2±10.2 | 20.4±10.8 | 0.02 |
| Creatinine (mg/dL) | 1.1±0.6 | 0.87±0.3 | 0.02 |
| C reactive protein (mg/dL) | 1.4±2.8 | 0.8±1.6 | 0.11 |
| Uric acid (mg/dl) | 8.6±5.1 | 5.1±3.9 | 0.04 |

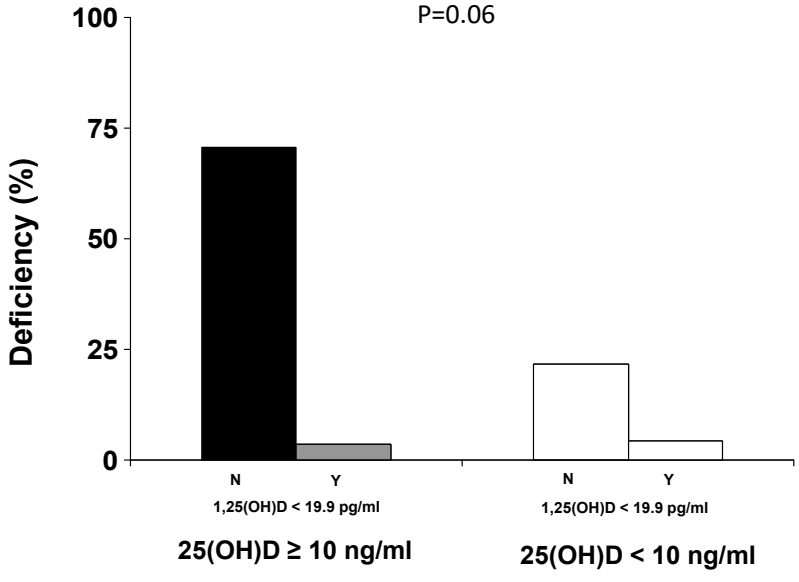
*CAD = Coronary Artery Disease; MI = Myocardial Infarction; PCI = Percutaneous Coronary Interventions; CABG = Coronary Artery Bypass Grafting; STEMI = ST-Elevation Myocardial Infarction; ACS = Acute Coronary Syndrome; CMD =Dilated Cardiomyopathy; LV = Left Ventricle; ACE = Angiotensin Converting Enzyme; ARB = Angiotensin Receptor Blockers;*

Table 2. Angiographic characteristics according to median values of 1,25(OH)2D

| Angiographic features | < 41.2 pg/ml (n=166) | ≥41.2 pg/ml (n=149) | P value |
|---|---|---|---|
| Left main disease (%)§ | 11.1 | 9.7 | 0.99 |
| LAD (%)§ | 83.3 | 79.2 | 0.67 |
| CX (%)§ | 59.7 | 51.4 | 0.40 |
| RCA (%)§ | 59.7 | 58.3 | 0.99 |
| Type C Lesion (%) | 54.8 | 51 | 0.57 |
| Lesion length (mm) | 23.5±13.3 | 22.7±13.4 | 0.63 |
| Percent stenosis (%) | 89.7±9.4 | 89.9±9.8 | 0.85 |
| Reference Diameter (mm) | 3.1±0.5 | 3±0.6 | 0.12 |
| Bifurcations (%) | 24.7 | 22.8 | 0.79 |
| Calcifications (%) | 17.5 | 12.8 | 0.27 |
| Chronic occlusion (%) | 16.4 | 16.1 | 0.99 |
| Restenosis (%) | 5.6 | 6.1 | 0.99 |
| Thrombus (%) | 9 | 11.4 | 0.58 |
| TIMI Flow | | | 0.31 |
| 3 | 75.3 | 69.1 | |
| 2 | 3 | 5.4 | |
| 1 | 1.2 | 0.7 | |
| 0 | 20.5 | 24.8 | |

Lower calcitriol was associated to diabetes (p=0.05), renal failure (p=0.02), hypertension (p=0.001), use of calcium-antagonists (p=0.001), diuretics (p=0.007), higher creatinine (p=0.02), uric acid (p=0.04), and worse glycemic and lipid profile. Angiographic characteristics did not substantially differ according to calcitriol median, as in Table 2.

Figure 1 Prevalence of 25 and 1,25(OH)2D deficiency in included population



37 patients (25.7%) had hypovitaminosis D, whereas 11 (7.9%) displayed calcitriol < 19.9 pg/ml (16.2% among patients with hypovitaminosis D and 4.9% with normal levels, p=0.06), as in Figure 1.

The independent predictors of 1,25(OH)2D deficiency were hypertension (OR[95%CI]=3.72[1.21-11.4], p=0.002), use of diuretics (OR[95%CI]=2.5[1.01-6.24], p=0.047), while negative predictor was the level of vitamin D (OR[95%CI]=0.930.89-0.98], p=0.008).

Figure 2 Linear relationship between 25 and 1,25(OH)2D

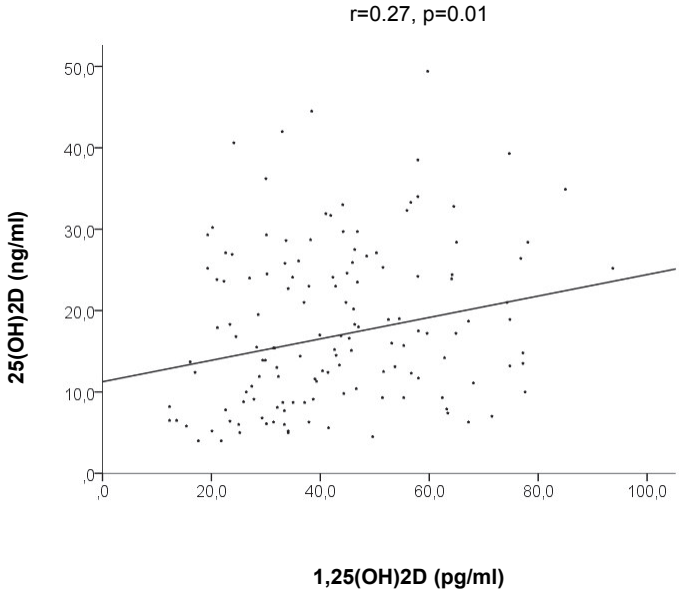
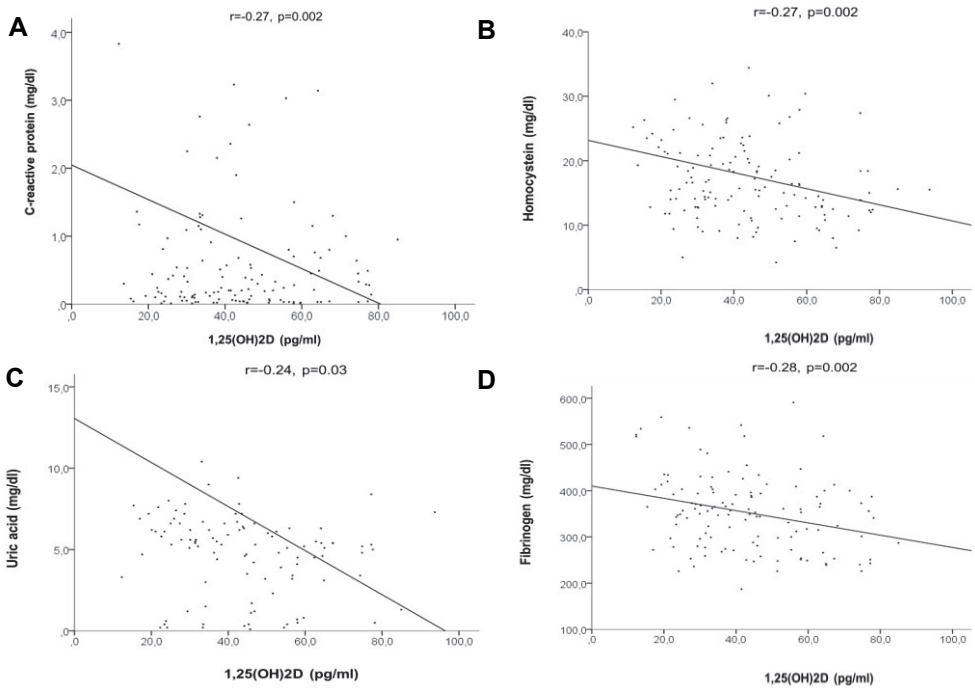
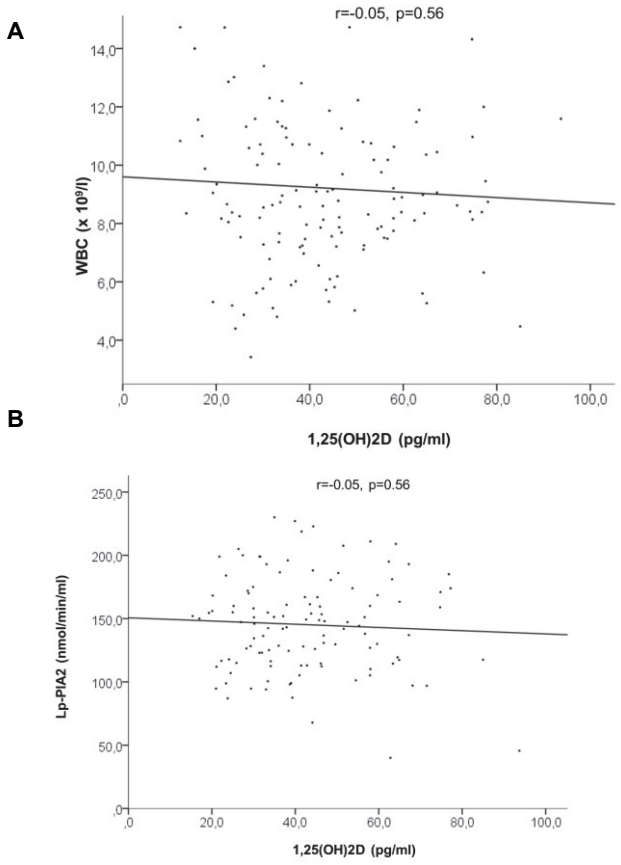



Figure 3. Linear relationship between 1,25(OH)2D and C-reactive protein (3A, upper left), Homocystein (3B, upper right), Uric acid (3C, lower left) and Fibrinogen (3D, lower right).



Calcitriol levels, in fact, directly related with the levels of vitamin D (r=0.27, p=0.01, Figure 2), whereas an inverse linear relationship was observed with major inflammatory and metabolic markers of cardiovascular risk, (C-reactive protein: r=-0.27, p=0.002; uric acid: r=-0.24, p=0.03; homocysteine: r=-0.27, p=0.002; fibrinogen: r=-0.28, p=0.002, Figure 3 A, B, and C), but for white blood cell count (r=-0.05, p=0.56) and Lp-PLA2 (r=-0.05, p=0.56), as in Figure 4A and B respectively.

**Figure 4** Linear relationship between 1,25(OH)2D and white blood cells (4A, upper graph), lipoprotein-associated phospholipase A2 (Lp-PlA2, 4B, lower graph).




## DISCUSSION

This is the first study conducted so far to evaluate the clinical determinants of calcitriol levels and their relationship with inflammatory and cardiovascular risk biomarkers among ACS patients undergoing early invasive management. Despite we observed a significant independent association of 1,2(OH)2D with vitamin D levels, its deficiency was much more infrequent as compared to hypovitaminosis D, suggesting

a potential “reserve” of vitamin D activation despite a deficiency of the substrate. Moreover, the prevalence of calcitriol deficiency was non-significantly lower in patients with 25(OH)D levels < 10 ng/ml, therefore suggesting the relevance of factors modulating the transformation of calcitriol and the importance of the direct dosing of the activated hormone rather that vitamin D. In addition, despite we included a high-risk ACS population, calcitriol related with several parameters mirroring the pro-inflammatory status and cardiovascular risk, therefore suggesting a more complex disease and potentially worse long-term outcomes.

Despite calcitriol represents the perpetrator of the effects of vitamin D, few studies have so far addressed the impact of its deficiency on the maintenance of the systemic homeostasis and in the pathogenesis of the several disorders linked to hypovitaminosis D (15,16). In fact, the relatively shorter plasmatic half-life of 1,25(OH)2D, only 12–36 h, in the human circulation as compared to its precursor has often been considered a barrier for its clinical application (17). However, 25(OH)D and 1,25(OH)2D cannot be considered equivalent measurements. Moreover, circulating calcitriol can be considered as being in a relatively steady state in healthy subjects (18) and, based on the development of more accurate methods for its measurement, raising interest has been addressed towards the consideration of the active form of vitamin D, and especially after the inconclusive results of the studies with 25(OH)D supplementation.

In fact, in a recent study on dialysis patients no change in the intestinal absorption of Ca was obtained after 3months of 20,000 IU/week of cholecalciferol therapy before the normalisation of 25D and improvement of 1,25D levels (19). In further studies, no effect on bone metabolism parameters was evident following cholecalciferol supplementation of dialysis patients (20).

A similar superiority of calcitriol above vitamin D has been documented in providing a cardioprotective, pleiotropic effect. In a recent study, Zitterman et al (11) documented among over 500 patients with heat failure that lower calcitriol was associated with a higher rate of coronary artery disease and

increased mid-term mortality. Moreover, in vitro study have shown that the active form of vitamin D induces a complex dual upregulation of endothelin and nitric oxide in cultured endothelial cells, reduces the expression of the angiotensin-1 receptor, improving endothelial function and preventing reactive oxygen species overproduction and downregulates hyperinsulinism and insulin resistance, lowering also the concentrations of various inflammatory markers including TNF-a, CRP and IL-6 in an overweight animal model (21-23).

In addition, calcitriol has been shown to raises the levels of IL-12, that is relevant to macrophage activity and atherogenesis (24) and to modulate the coagulation cascade, increasing thrombomodulin and down-regulating tissue factor (25). However, its role in coronary thrombosis has never been assessed.

Indeed, it could be hypothesized that the direct assessment of the levels of calcitriol could provide a more strict correlation with the pathogenesis of atherosclerotic lesions and their transient variations could favour plaques rupture, which represents the well-established causal mechanisms for acute coronary syndromes. Nevertheless, such hypothesis has never been considered so far in a dedicated clinical study.

We firstly report the prevalence and predictors of calcitriol deficiency in a consecutive cohort of ACS patients undergoing early invasive management. We documented that lower calcitriol levels were associated with main established cardiovascular risk factors, and in particular with hypertension, diabetes and worse lipid profile, but also with renal function, and with a non-significant trend for presentation with ST-segment elevation MI.

However, at multivariate analysis, only hypertension, use of diuretics and baseline levels of 25(OH)D emerged as independent predictors of calcitriol < 19.9 pg/ml, whereas no impact was played by age or gender, in accordance to the previous reports by Levin et al. (26) in an unselected cohort of 1814 subjects. However, in this study, 1,25(OH)2D levels were related to diabetic status and parameters of renal function, as glomerular filtration and albumin-to-creatinine levels, although they recruited a large

proportion of patients with kidney disease. In addition, oppositely to our results, the prevalence of calcitriol deficiency, in Levin et al. was much higher than calcitriol deficiency. However, about 35-40% of the study cohort was receiving a multivitamin supplementation, thus potentially accounting for the lower prevalence of hypovitaminosis D, as compared to our data.

In fact, similarly to our conclusion, in a previous study over 500 patients with different degrees of renal dysfunction (from no chronic kidney disease-CKD to haemodialysis), Pasquali et al. (27) documented that both vitamin D levels, renal function and parathyroid hormone could modulate the efficiency of the hydroxylation of 25(OH)D to calcitriol. In fact, the lowest absolute values were observed in haemodyalisis patients without vitamin D deficiency, as these patients cannot be expected to substantially increase circulating levels of calcitriol from damaged kidneys simply because of substrate availability. In comparison, replete CKD patients exhibited a hydroxylation efficiency that, on average, did not significantly differ from HD patients, suggesting that the system is blunted in spite of some residual renal function.

In our study, the rate of patients with inadequate calcitriol levels was less than 50% of the patients displaying sever vitamin D deficiency, suggesting the possibility to recruit a synthetic “reserve” for preserving the levels and function of the active hormone. In fact, the efficiency of 1,25(OH)2 D hydroxylation, however, can be rapidly increased by paracrine and auto-regulatory mechanisms, stimulating a local activity of the 1-α-hydroxylase in presence of high PTH, low calcium or inflammatory factors and cytokines. In fact, Pasquali et al. (27) showed in subjects with no CKD, that the efficiency of calcitriol production was linearly related to the levels of substrate, but became an exponential increment when 25(OH)D levels declined below 20 ng/ml. On the contrary, such reserve was not observed in presence of renal dysfunction, confirming that kidney activity and vitamin D levels represent the principal determinants of calcitriol production.

Nevertheless, in our study, use of diuretics, but not the levels of creatinine or the history of CKD emerged as independent predictors of 1,25(OH)2D deficiency. Indeed, vitamin D transformation is a tubular process, therefore being potentially less related with the levels of renal clearance and glomerular filtration rate (GFR). Consistently, in a previous report, Prince et al. (28) documented a preserved calcitriol synthesis despite GFR reduction in patients with moderate CKD.

Similar conclusion was also reached by Pasquali et al., (27) showing an exponential growth in vitamin D hydroxylation, among haemodyalisis patents, underlining the potential role of extra-renal production of calcitriol.

Such paracrine activation of vitamin D could play an intriguing role in preventing the acute inflammatory processes leading to the instabilization and rupture of a plaque and therefore to the onset of acute coronary syndromes. In fact, we observed a direct linear correlation of calcitriol with several biomarkers of the inflammatory and thrombotic process, even in a selected population of high-risk ACS patients, where an elevation of such parameters could be expected in all patients. However, 1,25(OH)2D did not correlate with the levels of Lp-PlA2 and white blood cells, suggesting that in the acute context the anti-inflammatory effect of vitamin D could be elicited more on the humoral that on the cellular-mediated response. Nevertheless, such hypothesis certainly needs further confirmations in larger dedicated studies, aiming at the exact definition of the pathophysiological mechanisms and the prognostic relevance of our observations, potentially offering new targets for the treatment and risk stratification of ACS patients.

*Study limitations*

The first limitation to our study is the relatively low sample of included patients, therefore, being more subject to any difference due to the non-randomized design of the study. In particular, the differential distribution of cardiovascular risk factors could have partially represented a confounder.

Moreover, we restricted our analysis to higher-risk ACS patients, in absence of a control population. Nevertheless, such recruitment was planned on purpose, since no data have been so far reported for calcitriol in an acute cardiovascular setting, representing on the contrary a subset of patients that could achieve the greatest benefits from the restoration of the cardioprotective effects of vitamin D.

Finally, we did not collect follow-up data, especially in patients undergoing coronary angioplasty, and thus cannot exclude an impact of 1,25(OH)2D of the progression of CAD, recurrent cardiovascular events and the risk of adverse events after PCI.

## CONCLUSION

The present study shows that among ACS patients, hypovitaminosis D is frequent, whereas being associated with sufficient levels of calcitriol in the majority of cases. We identified hypertension, use of diuretics and lower vitamin D as independent predictors of 1,25(OH)2D deficiency. Moreover, we documented a significant inverse relationship of calcitriol with C-reactive protein, uric acid, homocysteine, and fibrinogen, inflammatory and metabolic biomarkers strictly linked to cardiovascular risk

# BIBLIOGRAPHY


1. Pilz S, Trummer C, Pandis M, Schwetz V, Aberer F, Grübler M, Verheyen N, Tomaschitz A, März W. Vitamin D: Current Guidelines and Future Outlook. Anticancer Res. 2018;38(2):1145-1151

2. Mozos I, Marginean O. Links between Vitamin D Deficiency and Cardiovascular Diseases. Biomed Res Int. 2015;2015:109275

3. Kunadian V, Ford GA, Bavamia B et al. Vitamin D deficiency and coronary artery disease: A review of the evidence. Am Heart J 2014;167:283-91

4. Elamin MB., Abu Elnour NO., Elamin KB., Fatourechi MM., Alkatib AA., Almandoz JP., Liu H., Lane MA., Mullan RJ., Hazem A., Erwin PJ., Hensrud DD., Murad MH., Montori VM. Vitamin D and cardiovascular outcomes: a systematic review and meta-analysis. Journal Clinical of Endocrinology & Metabolism 2011;96:1931-42

5. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. BMJ. 2014;348:g2035

6. Schneider ALC, Lutsey PL., Selvin E. Vitamin D, vitamin D binding protein gene polymorphisms, race and risk of incident stroke: The Atherosclerosis Risk in Communities (ARIC) study. European Journal Neurology. 2015;22:1220-1227

7. Daffara V, Verdoia M, Rolla R, Nardin M, Marino P, Bellomo G, Carriero A, De Luca G; Novara Atherosclerosis Study Group (NAS). Impact of polymorphism rs7041 and rs4588 of Vitamin D Binding Protein on the extent of coronary artery disease. Nutr Metab Cardiovasc Dis. 2017;27(9):775-783

8. Zittermann A, Schleithoff SS, Frisch S, Götting C, Kuhn J, Koertke H, Kleesiek K, Tenderich G, Koerfer R. Circulating calcitriol concentrations and total mortality. Clin Chem. 2009;55(6):1163-70

9. Mawer EB, Schaefer K, Lumb GA, Stanbury SW. The metabolism of isotopically labelled vitamin D3 in man: the influence of the state of vitamin D nutrition. Clin Sci 1971;40:39 –53

10. Juttman jr, Buurman cj, De Kam e, Visser ti, Birkenhager jc: Serum concentrations of metabolites of vitamin D in patients with chronic renal failure (CRF). Consequences for the treatment with l-a-hydroxy-derivatives. C/in Endocrinol 1981: 14:225—236,

11. Richart T, Li Y, Staessen JA. Renal versus extrarenal activation of vitamin D in relation to atherosclerosis, arterial stiffening, and hypertension. Am J Hypertens. 2007;20:1007–1015

12. De Luca G, Secco GG, Santagostino M, Venegoni L, Iorio S, Cassetti E, Verdoia M, Coppo L, Di Mario C, Bellomo G, Marino P; Novara Atherosclerosis Study Group (NAS). Uric acid does not affect the prevalence and extent of coronary artery disease. Results from a prospective study. Nutr Metab Cardiovasc Dis. 2012;22:426-33.

13. Holick MF. Vitamin D Deficiency. N Engl J Med. 2007;357:266-281.

14. Davidson MH1, Corson MA, Alberts MJ, Anderson JL, Gorelick PB, Jones PH, Lerman A, McConnell JP, Weintraub HS.Consensus panel recommendation for incorporating lipoprotein-associated phospholipase A2 testing into cardiovascular disease risk assessment guidelines. Am J Cardiol. 2008 16;101(12A):51F-57F.

15. Dong J, Wong SL, Lau CW, Lee HK, Ng CF, Zhang L, Yao X, Chen ZY, Vanhoutte PM, Huang Y. Calcitriol protects renovascular function in hypertension by down-regulating angiotensin II type 1 receptors and reducing oxidative stress. Eur Heart J. 2012;33(23):2980-90

16. Takeda M, Yamashita T, Sasaki N, Nakajima K, Kita T, Shinohara M, Ishida T, Hirata K. Oral administration of an active form of vitamin D3 (calcitriol) decreases atherosclerosis in mice by inducing regulatory T cells and immature dendritic cells with tolerogenic functions. Arterioscler Thromb Vasc Biol. 2010 Dec;30(12):2495-503

17. Jongen MJ, Bishop JE, Cade C, Norman AW. Effect of dietary calcium, phosphate and vitamin D deprivation on the pharmacokinetics of 1,25-dihydroxyvitamin D3 in the rat. Horm Metab Res 1987;19:481

18. Zittermann A, Heer M, Caillot-Augusso A, Rettberg P, Scheld K, Drummer C, et al. Microgravity inhibits intestinal calcium absorption as shown by a stable strontium test. Eur J Clin Invest 2000;30:1036–43

19. Armas LA, Zena M, Lund R, Heaney RP. Calcium absorption response to cholecalciferol supplementation in hemodialysis. Clin J Am Soc Nephrol. 2013 ;8(6):1003-8

20. Hewitt NA, O'Connor AA, O'Shaughnessy DV, Elder GJ. Effects of cholecalciferol on functional, biochemical, vascular, and quality of life outcomes in hemodialysis patients. Clin J Am Soc Nephrol. 2013 Jul;8(7):1143-9

21. Fryer RM, Rakestraw PA, Nakane M, Dixon D, Banfor PN, Koch KA, Wu-Wong JR, Reinhart GA. Differential inhibition of renin mRNA expression by paricalcitol and calcitriol in C57/BL6 mice. Nephron Physiol. 2007;106(4):p76-81

22. Alkharfy KM, Al-Daghri NM, Yakout SM, Ahmed M. Calcitriol attenuates weight-related systemic inflammation and ultrastructural changes in the liver in a rodent model. Basic Clin Pharmacol Toxicol. 2013 Jan;112(1):42-923.

23. Muller K, Haahr PM, Diamant M, Rieneck K, Kharazmi A, Bendtzen K. 1,25-dihydroxyvitamin D3 inhibits cytokine production by human blood monocytes at the post-transcriptional level. Cytokine
1992;4:506 –12

24. Uyemura K, Demer LL, Castle SC, Jullien D, Berliner JA, Gately MK, Warrier RR, Pham N, Fogelman AM, Modlin RL. Cross-regulatory roles of interleukin (IL)-12 and IL-10 in atherosclerosis. J Clin Invest. 1996 May 1;97(9):2130-8

25. Martinez-Moreno JM, Herencia C, Montes de Oca A, Muñoz-Castañeda JR, Rodríguez-Ortiz ME, Díaz-Tocados JM, Peralbo-Santaella E, Camargo A, Canalejo A, Rodriguez M, Velasco-Gimena F, Almaden Y. Vitamin D modulates tissue factor and protease-activated receptor 2 expression in vascular smooth muscle cells. FASEB J. 2016;30(3):1367-76

26. Levin A, Bakris GL, Molitch M, Smulders M, Tian J, Williams LA, Andress DL. Prevalence of abnormal serum vitamin D, PTH, calcium, and phosphorus in patients with chronic kidney disease: results of the study to evaluate early kidney disease. Kidney Int. 2007;71(1):31-8

27. Pasquali M, Tartaglione L, Rotondi S, Muci ML, Mandanici G, Farcomeni A, Marangella M, Mazzaferro S. Calcitriol/calcifediol ratio: An indicator of vitamin D hydroxylation efficiency? BBA Clin. 2015;3:251-6

28. Prince RL, Hutchison BG, Kent JC, Kent GN, Retallack RW. Calcitriol deficiency with retained synthetic reserve in chronic renal failure. Kidney Int. 1988 ;33(3):722-8

# Conclusions and future perspectives

Vitamin D deficiency represents a pandemic social problem affecting over 1 billion of people worldwide, although its clinical relevance is still largely neglected (1).

Several studies have documented that the maintenance of adequate levels of vitamin D offers benefits in terms of survival, quality of life and for the maintenance of "house-keeping" homeostatic processes in different organs and tissues, including the cardiovascular system (2,3).

However, the exact threshold for the definition of the optimal levels of vitamin D required for the achievement of the cardioprotective effects is still debated. In fact, while a circulating 25(OH)D < 10 ng/ml is universally accepted as a severe deficiency, it is still unclear whether the cut-off for sufficiency is to be settled at 20 or 30 ng/ml (4).

In Chapter 2, we documented a progression of the risk of coronary artery disease with the lowering of vitamin D levels, although its prevalence and extent were significantly enhanced only for patients with levels < 10 ng/ml.

In addition, the present thesis documented, in Chapter 8, that, even among subjects with vitamin D < 10 ng/ml, yet the levels of circulating active hormone could be preserved, therefore pointing at the superiority of the direct evaluation of calcitriol levels rather than vitamin D precursors.

Indeed, the definition of the complex regulatory mechanisms responsible for the homeostasis of vitamin D levels still needs further clarification (5). In fact, both genetic factors and several clinical conditions, despite not conditioning the measured circulating 25(OH)D, can modulate its bioavailability and effects, therefore translating into cardiovascular consequences.

In Part 3 in fact, we showed that both genetic variants of vitamin D binding protein (VDBP) and of the 24-α hydroxylase responsible for vitamin D inactivation (CYP24A1), can be held responsible for the clinical manifestations of vitamin D deficiency, against comparable levels of vitamin D. In fact, carriers

of the G allele of rs7041 of VDBP; a variant associated with an enhanced binding of the vitamin to its transport-protein, displayed an increased thrombogenicity despite dual antiplatelet therapy (DAPT), only when this genetic status was associated with vitamin D deficiency.

Moreover, variability in the levels of the active hormonal form, calcitriol, could affect coronary calcifications and the inflammatory status. In Chapter 7, in fact, we showed that the wild-type genotype for rs2762939 of CYP24A1 were associated with coronary calcifications, potentially mediated by an increased inactivation of 1,25(OH)2D due to a higher enzymatic activity. In addition, in Chapter 8, we reported that activated vitamin D in ACS patients could be conditioned not only by the levels of its precursor, but also by other factors as hypertension and renal dysfunction. Indeed, the kidney represents the principal site of activation of vitamin D, and mainly at a tubular level, therefore explaining the reason for our positive association with the use of diuretics rather than with serum creatinine, instead representing a glomerular process. In addition, hypertension, by increasing the wall shear stress, could favor the induction of the pathway responsible for the paracrine production of calcitriol in the endothelium of the whole arterial system, thus increasing its circulating levels. On the contrary, an extremely focused vascular damage, as in case of an acute coronary syndrome, being dependent on the instabilization of a single atherosclerotic lesion, could also stimulate a local production of 1,25(OH)D, that could nevertheless result in too modest variations to be apparent at a systemic level. Such hypothesis, as much as the common pathogenesis of the spectrum of acute coronary events, could certainly explain our observation of similar levels of 1,25(OH)2D in different ACS types.

Therefore, present thesis directly points out at calcitriol as a marker of cardiovascular disease and a potential new target for a pharmacological approach to cardiovascular prevention.

In fact, 1,25(OH)2D, being linked to a pro-inflammatory and pro-thrombotic status and to vascular wall degeneration and remodeling, could represent an early predictor of cardiovascular risk, even in subjects without established coronary atherosclerosis.

In addition, the definition of the optimal levels of calcitriol for cardiovascular prevention could allow to optimize the therapy and to define which patients could mostly benefit from its supplementation, and especially for high-cardiovascular risk patients, as in the context of an ACS.

Indeed, the trials conducted so far on the topic are largely inadequate for answering to this issue, not being powered for the evaluation of cardiovascular endpoints and often failing in the restoration of adequate levels of vitamin D (6,7). The ongoing the VITamin D and OmegA-3 TriaL (VITAL) trial (8) will certainly shed more light on the topic, by enrolling 25,874 men and women across the U.S. taking daily dietary supplements of vitamin D3 or omega-3 fatty acids for the reduction of the risk of cancer, heart disease, and stroke.

Nevertheless, the majority of the studies were conducted with inactive 25(OH)D, then being subject to a differential activation and potentially not resulting in sufficient hormonal levels. In fact, the studies with direct calcitriol replacement have being conducted so far only in patients with renal failure, providing promising results, (9,10) that however, will certainly deserve confirmations in a more general population.

## References


1. Holick MF. The vitamin D deficiency pandemic: Approaches for diagnosis, treatment and prevention. Rev Endocr Metab Disord. 2017;18(2):153-165

2. Gil Á, Plaza-Diaz J, Mesa MD. Vitamin D: Classic and Novel Actions. Ann Nutr Metab. 2018;72(2):87-95

3. Norman PE, Powell JT. Vitamin D and cardiovascular disease. Circ Res. 2014;114(2):379-9

4. Lugg ST, Howells PA, Thickett DR. Optimal Vitamin D Supplementation Levels for Cardiovascular Disease Protection. Dis Markers. 2015;2015:864370

5. Barry EL, Rees JR, Peacock JL, et al. Genetic Variants in CYP2R1, CYP24A1, and VDR Modify the Efficacy of Vitamin D3 Supplementation for Increasing Serum 25-Hydroxyvitamin D Levels in a Randomized Controlled Trial. The Journal of Clinical Endocrinology and Metabolism. 2014;99(10):E2133-E2137.

6. Theodoratou E, Tzoulaki I, Zgaga L, Ioannidis JP. Vitamin D and multiple health outcomes: umbrella review of systematic reviews and meta-analyses of observational studies and randomised trials. BMJ. 2014;348:2035

7. Rejnmark L, Bislev LS, Cashman KD, et al. Non-skeletal health effects of vitamin D supplementation: A systematic review on findings from meta-analyses summarizing trial data. Slominski AT, ed. PLoS ONE. 2017;12(7):e0180512..

8. Manson JE, Bassuk SS, Lee IM, Cook NR, Albert MA, Gordon D, Zaharris E, Macfadyen JG, Danielson E, Lin J, Zhang SM, Buring JE. The VITamin D and OmegA-3 TriaL (VITAL): rationale and design of a large randomized controlled trial of vitamin D and marine omega-3 fatty acid supplements for the primary prevention of cancer and cardiovascular disease. Contemp Clin Trials. 2012;33(1):159-71.

9. Verouti SN, Tsoupras AB, Alevizopoulou F, Demopoulos CA, Iatrou C. Paricalcitol effects on activities and metabolism of platelet activating factor and on inflammatory cytokines in hemodialysis patients. Int J Artif Organs. 2013;36(2):87-96

10. Shoben AB, Levin G, de Boer IH, et al. Variation in Oral Calcitriol Response in Patients With Stage 3-4 CKD. American Journal of Kidney Diseases. 2012;59(5):645-652.