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COORDINATOR: PROF. EMANUELE ALBANO

SCUOLA ALTA FORMAZIONE

PhD project

Title:

Paracrine modulation of 1,25(OH)2D levels in cardiovascular system.

Genetic regulation, impact on degenerative aortic valve disease, inflammation, platelet function and periprocedural myocardial injury after percutaneous coronary interventions.

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Introduction

Vitamin D is a secosteroid and a hormonal precursor, whose hydroxylyzed, activated form (1,25(OH)2D or calcitriole) represents the main modulator of calcium and bone homeostasis. Nevertheless, vitamin D also acts as a transcriptional factor that, through its receptor (VDR), regulates the expression of almost 3% of human genome, displaying its effects on several districts including the muscles, the liver, kidneys, the immune and the nervous system, the vessel wall and platelets (1,2).

Vitamin D introduction is partly dietary and in part derives from a cutaneous, non-enzymatic transformation of circulating precursors, produced by sun exposure. This form of vitamin D undergoes then two steps of hydroxylation in the liver and kidneys to obtain the fully active hormonal form.

Vitamin D deficiency has achieved, in the last years, a dramatic prevalence in Western counties, ranging over 50% of prevalence in the population, (3), as a consequence of the progressive ageing and the increased frequency of chronic disorders as renal failure.

Recent attention has been addressed to the cardiovascular effects of vitamin D, (4), as its deficiency has been involved in the pathogenesis of hypertension, diabetes mellitus, metabolic disorders, but it has also been associated to vascular wall calcifications, vascular intima thickening, ventricular hypertrophic remodeling, and thrombotic disorders (5,6).

Different studies and a recent meta-analysis have demonstrated the clear negative prognostic impact of hypovitaminosis D on all-cause and cardiovascular mortality, (7), showing an inverse linear relationship with an increase in cardiovascular risk for every 10 ng/ml

reduction in 25OHD. However, so far clinical trials providing vitamin D supplementation have failed to demonstrate any significant benefit in cardiovascular outcomes (8).

Several explanations have been provided for such findings and particular attention has been focused on the role of genetics.

In fact, two common genetic variants of Vitamin D Binding Protein (VDBP) have been held responsible for more than 10% of the interindividual variability in the circulating levels of 25OHD, conditioning its bioavailability and peripheral effects (9).

Variations in the DBP originally referred to as GC1F, GC1S, and GC2 were first reported more than 50 years ago and may be associated with changes in binding affinity or serum concentration of DBP. The protein variants are now recognized as resulting from polymorphisms in the DBP binding protein gene GC. The phenotypic variations in the DBP amino acid sequence are distinguished by single nucleotide polymorphisms (SNPs) rs7041 and rs4588, positioned at codons 416 (GAT→GAG, Asp→Glu) and 420 (ACG→AAG, Thr→Lys) of exon 11 of the GC gene. Blacks and Asians are more likely to carry GC1F DBP (wild-type alleles) , which has the highest affinity for 25(OH)D and is associated with low DBP levels. Whites are more likely to carry GC1S DBP (rs4588 minor allele carriers); but also GC2 (rs7041 minor allele carriers), which has a lower affinity for 25(OH)D and is associated with higher DBP levels, is frequently found in whites.

In addition, five single nucleotide polymorphisms have been described for vitamin D receptor, partially combined among them in consequence of linkage disequilibrium, to generate specific haplotypes with functional consequences on the receptor (10).

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 calcitriole 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 (11). *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 calcitriole).

Moreover, genetic variants of these hydroxylising enzymes have been described, although their impact on vitamin D signaling is still matter of debate (12).

A more intriguing hypothesis, however, recently proposes that many effects of 25OHD could be the consequence of a local, autocrine or paracrine activation of vitamin D (13). 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 (14).

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 (15). 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 (16).

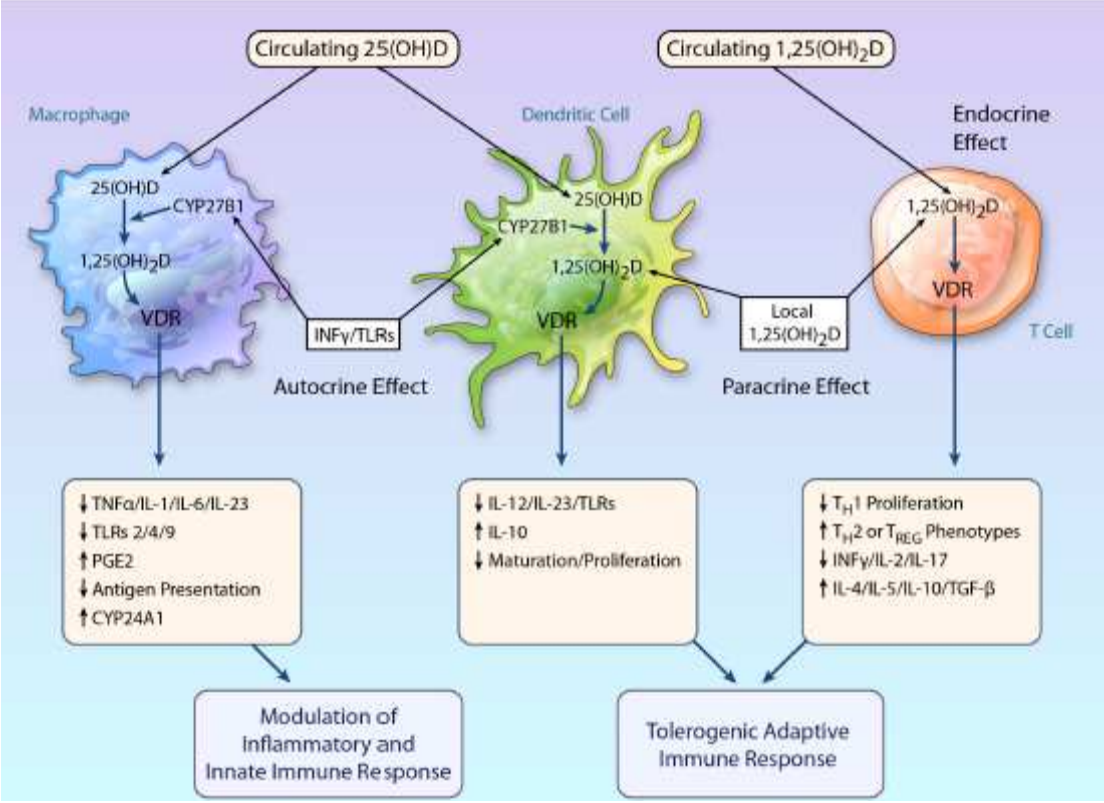

Figure 1: Autocrine and paracrine effects of vitamin D on the immune system (Norman et al, Circ. Res 2014)

In addition, 1,25(OH)2D can contrast the systemic effects of the renin-angiotensin-aldosterone cascade (RAS), therefore regulating blood pressure, but also its can also prevent the activation of RAS at a local level, in macrophages and cardiomyocytes, and lower the expression of angiotensin-II in endothelial cells, that are known to condition cardiac hyperthrophy, fibrosis and endothelial dysfunction, leading to the development of the atheromasic plaque (17).

Merke *et al.* have also demonstrated that the endothelial stress can rapidly induce the expression of VDR and CYP27B1, suggesting a paracrine role of 1,25(OH)2D. In fact, this

activation of vitamin D can translate into an increased release of nitric oxide from endothelial cells, an enhanced flow-mediated vasodilatation and also favors the production of growth factors inducing vessel wall healing (18,19).

Finally, a paracrine epigenetic role has been proposed for VDR, that can modulate within seconds-minutes the trans-membrane transportation of calcium, thus exerting regulatory effects on intracellular signaling, on the proteins of the sarcomere (for muscular contraction) and microtubules (for the release of the granules of inflammatory cells and platelets) (20,21). In fact, in previous studies conducted in a murine model, vitamin D has demonstrated a role in preventing the acute damages of hypoxia, cellular apoptosis and oxidative stress (22). Moreover, the loss of function of VDR has been associated to a higher occurrence of ventricular fibrillation in rats where an ischemia-reperfusion myocardial injury was experimentally induced, while the administration of vitamin D could prevent the onset of arrhythmias, suggesting a short term anti-ischemic action of calcitriole (23).

Nevertheless, the cardiovascular impact of these “rapid” effects of vitamin D is poorly understood. Anyhow, a relevant role could be hypothesized especially in the context of acute coronary syndromes, where the pro-thrombotic, pro-inflammatory and pro-oxydant setting might enhance the paracrine activity of vitamin D. In addition, no study has so far addressed those genetic factors that could condition an interindividual difference in the bioavailability and local effectiveness of 1,25(OH)2D in coronary arteries, therefore providing potential explanation for the negative findings of previous trials with vitamin D.

Study aims

Aim of present project is to evaluate:

1) the impact of genetic variants of vitamin D binding protein (VDBP), VDR and and CYP27B1 on the systemic levels of vitamin D (either 25OHD and 1,25(OH)2D) and their cardiovascular consequences on:

- coronary artery disease,
- carotid intima-media thickness,
- aortic valve degeneration;
- platelet reactivity;

2) the relationship between the systemic and intracoronary levels of 25OHD and 1,25(OH)2D and their transient modifications in patients undergoing percutaneous coronary interventions (PCI), in order to define:

- whether interindividual differences in the paracrine production of vitamin D can impact on clinical presentation in acute coronary syndrome patients and whether these variations are influenced by VDBP, VDR and CYP27B1 polymorphisms;
- whether PCI can induce a paracrine activation of 1,25(OH)2D ;
- whether a differential activation of vitamin D can translate into consequences on periprocedural myocardial infarction.

Methods

Population in study

In a first part of the study, consecutive patients undergoing coronary angiography at the Emodinamica Universitaria, Ospedale "Maggiore della Carità", Novara, Italy will be included. Patients receiving vitamin D supplementation at admission or those whose vitamin D status was unavailable will be excluded as well as those who will refuse to sign written informed consent. All demographic and clinical data will be prospectively collected in a dedicated database, protected by password. For all patients we will collect before the angiography all clinical data and a fasting blood sample for routine chemistry, genetics and cardiac biomarkers (Troponin I and CK MB).

For the second part of the study, only patients with acute coronary syndrome (ACS) at presentation, undergoing coronary angiography and subsequent PCI of critical coronary stenoses defined as "culprit" will be consecutively enrolled.

ACS patients will be considered for unstable angina and myocardial infarction ST-segment elevation (STEMI) or non- ST segment elevation (NSTEMI), defined as chest pain lasting more than 5 minutes, with or without elevation of cardiac biomarkers beyond the upper limit of normal (ULN) (respectively 0,04 µg/l for Troponin I and 5,00 µg/l for CK-MB), with or without ECG changes.

Coronary angiography and Quantitative Coronary Angiography (QCA)

Coronary angiography (carried our by Siemens AXIOM ARTIS dTC, Erlangen, Germany) will be routinely performed by the Judkins technique using 6-French right and left heart catheters. Quantitative coronary angiography (QCA) will beperformed by two experienced

interventional cardiologists, by an automatic edge-detection system for Quantitative Coronary Angiography (Siemens Acom Quantcor QCA, Erlangen, Germany). After a visual inspection of the coronary artery, the frame of optimal clarity will be selected, showing lesion at maximal narrowing and arterial silhouette in sharpest focus. After the calibration of guiding catheter, analyzed arterial segment with coronary lesion will be 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 will be measured. Significant CAD will be defined as the presence of at least 1 coronary stenosis more than 50%. Severe CAD will be 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 will be considered as significantly diseased vessel. In previously bypassed patients, both native arteries and grafts will be taken into account in the evaluation of extension of coronary artery disease (number of diseased vessels).

Coronary angioplasty will be performed with standard techniques. Use of stents, type of stents and stent implantation techniques, as much as the use of directional or rotational atherectomy, intravascular ultrasound (IVUS), glycoprotein IIb-IIIa inhibitors, will be left at the discretion of the operators. All patients will receive a dual antiplatelet therapy including Acetylsalicylic acid (ASA) 100 to 160 mg and an oral ADP-antagonists starting from the time of hospitalization or before angioplasty .

Biochemical measurements

Blood samples will be 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 will be determined by standard methods. Vitamin D dosing (25OHD and 1,25(OH)2D) will be performed by chemiluminescence method through LIAISON® Vitamin D assay (Diasorin Inc). The normal range for 25OHD levels in our laboratory is from 30 to 100 ng/ml, according to literature reference. Severe hypovitaminosis D is considered for values < 10 ng/ml as previously reported (24).

Paracrine production of 1,25(OH)2D

The paracrine production of vitamin D will be assessed in a sample derived from the part of the coronary artery distal to the "culprit" lesion, that will be obtained from the guiding catheter during PCI by the use of an over-the-wire balloon.

The levels of 25,OHD and 1,25(OH)2D will be measured as previously described and compared with a contextual sample derived from systemic circulation (in aorta). The dosing will be performed before and after PCI, to assess the paracrine response induced by the procedural endothelial damage.

Genetic polyorphisms in vitamin D regulation

Several single nucleotide polymorphisms (SNP) are associated to the regulation and metabolism of vitamin D.

In present study we will address:

- the two most common SNPs of vitamin D binding protein (VDBP), rs7041 and rs4588, that have been associated with variations in the circulating levels and the bioavailability of vitamin D and with the manifestations of vitamin D deficiency, in both bone mineral status and cardiovascular system (25,26).

- the most common SNPs of Vitamin D receptor (VDR), and in particolar the substitution *Fok-I* (rs10735810/rs2228570 C/T), located at the 3' part of the 3rd exon, that has been associated to diabetic nephropathy and melanoma, with a minor allele frequency (MAF (T)) of 37.9% in Caucasian population (27). In addition, we will evaluate the SNP *BsmI* (rs1544410 G/A), located in the 3' UTR, where the allele G (MAF 38-46%) has been shown to reduce the stability of the transcript, thus translating into a lower production of receptors and a reduced effectiveness of vitamin D (28). The latter variant is in linkage disequilibrium with *ApaI* and rs731236 (*Taq-I*), that will be, therefore, not evaluated.

- the gene coding for CYP27B1, the enzyme activating calcitriole, is regulated by VDR, that inhibits the production of the 1-α hydroxylase in presence of 1,25(OH)2D and induces it in absence of its ligand. We will analyze the SNP rs10877012 (G/T, MAF (T) =0.35) located in the promoter of the gene, thus conditioning the transcription of the enzyme.

Genetic analysis

Genomic DNA will be obtained from 200 μl of whole blood through a dedicated kit (GenElute™ Blood Genomic DNA, Sigma Aldrich). The genetic analysis will be performed by PCR-RFLP.

We will amplify the region of interest of the genomic DNA, by a Polymerase Chain Reaction that will be performed with dedicated primers in a Thermal cycler instrument Applied Byosistem T2720. A 3 step reaction will be planned: a step for activation of the enzyme, a subsequent amplification phase (including annealing of the primers, at a melting temperature varying according to the primer pair) and a final elongation phase. (to allow the extension of eventual uncompleted segments). A sample including no genomic DNA (negative control) will be added to every reaction.

The PCR product will be digested by dedicated restriction enzymes, allowing to selectively recognize the SNP in evaluation (either the wild-type or the mutated sequence). The product of the digestion will undergo electrophoretic run on agarose gel, an subsequent analysis by UV photography, in order to identify, according to the pattern of the segments, the genetic status.

For VDBP rs7041 and rs4588, a unique primer pair is currently in use. The forward sequence is (5'->3') 5'-GACTTCCAATTCAGCAGCGA-3', whilst the reverse 5'-CCCTCCACTTAACATGGCAG-3', resulting in a PCR product of 403 bp, that is then digested by HaeIII and StyI enzymes, that can generate 2 restriction fragments in presence of the minor allele.

Vitamin D and platelet aggregation

Several studies have suggested a role of vitamin D in modulating platelet function. We will assess the impact of 25 and 1,25(OH)2D on platelet reactivity and the effectiveness of antiplatelet agents.

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 or prasugrel 5 or 10 mg od) will be scheduled for chemistry and platelet function tests evaluation at 30-90 days from discharge.

Platelet aggregation will be measured by on whole blood, by impedance aggregometry (Multiplate®- multiple platelet function analyser; Roche Diagnostics AG) For Multiplate a whole blood sample will be stored in Vacutainer standard lithium heparin tubes and analyzed within 1-2 hours from collection. Tests with different agonists will be performed:

arachidonic acid (AA), collagen, ADP and prostaglandin E1 and thrombin receptor activating peptide (TRAP-6). The results will be expressed as arbitrary Aggregation Units (AU) and plotted against time, defining platelet function as the area under curve (AUC or AU*min). HRPR will be considered for AU*min values above lower limit normal for ASA (HAPR), [range: 862 - 1344] or after ADP stimulation [range: 417 - 1030] respectively (29). The previously reported (30) cut-off of > 468 AU*min (46 U) will also be applied to define poor clopidogrel/ticagrelor responders. The test will be repeated in patients with HRPR to confirm the findings.

Periprocedural effects of Vitamin D : modulation of 1,25(OH)2D and periprocedural myocardial injury

Periprocedural myocardial infarction (PMI) still represents a relatively common complication of PCI, occurring in a range between 5 to 20% of patients, with a negative prognostic impact. Several mechanisms are implied in PMI, including distal embolization, side-branch occlusion or coronary dissection, however myocardial injury can also occur after apparently uneventful PCI procedures in case of failure to obtain adequate myocardial perfusion. Microvascular obstruction by neutrophil and platelet plugs, local vasoconstriction and myocardial edema have been claimed for these silent events. (31,32).

The anti-inflammatory and anti-thrombotic effects of vitamin D might, therefore, prevent those microvascular alterations of the coronary flow and favor and anti-ischemic effect on cardiomiocytes, preventing a periprocedural myocardial damage.

In every patient, cardiac biomarkers (Troponin I and CK MB) will be measured at baseline, before coronary revascularization, and at intervals at 6, 12, 24 and 48 h after PCI.

We will define periprocedural MI as CK-MB mass release > 3 times the upper limit normal (ULN) or an increase by 50% of baseline if already elevated, but stable or falling, at the time of the procedure. We will consider myonecrosis, for a periprocedural increase in troponin I > 3 x ULN or an increase by 50% of the pre-procedural value, if > 0.04 ng/ml.

In addition, in accordante to the EEuropean Society of Cardiology (ESC) guidelines for the definition of myocardial infarction, the onset of symptoms or ECG change. s will be evaluated

The occurrence of a periprocedural myocardial injury will be assessed in function of the pre and post-PCI systemic and intracoronary levels of vitamin D and 1,25(OH)2D, and according to genetic status.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 statistical package. Continuous data are expressed as mean $\pm$ SD and categorical data as percentage. Analysis of variance and the chi-square test (or Fisher-test) will be used for continuous and categorical variables, respectively. Linear regression analysis will be applied for relating continuous variables with vitamin D levels.

Multiple logistic regression analysis will be performed to evaluate the relationship between vitamin D levels and coronary artery disease, periprocedural myocardial necrosis or infarction and the prevalence of HRPR after dual antiplatelet therapy, after correction for

clinical and angiographic significant differences that will be entered in the model in block. A p value <0.05 will be considered statistically significant.

The sample size of the study is calculated for the primary endpoint (impact of vitamin D genetics on coronary artery disease), whereas for other endpoints, due to the multiple comparisons and the lack of literature providing a potential reference on the expected differences, the fraction of the total population in study reflecting the inclusion criteria will be analyzed, with an established minimal study population of 100 patients.

For the primary endpoint, on the basis of a CAD prevalence of 70% an increased absolute risk of 7% (between 70% and 77%) will be considered clinically significant. Based on a MAF around 35% for the less frequent polymorphism, with a statistical power of 80% and alpha error of 0.05, the estimated sample size for our study population is 1274 patients, that will nevertheless be extended to 1500 patients in order to include a larger population for secondary endpoints.

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Congressual facts

ESC 2015 – MODERATED POSTER PRESENTATION (August 2015): Barbieri L, ***Verdoia M****, Marino P, Suryapranata H, De Luca G. "Contrast volume to Creatinine Clearance ratio for the prediction of Contrast Induced Nephropathy in patients undergoing coronary angiography or percutaneous intervention"*

ESC 2015 – MODERATED POSTER PRESENTATION (August 2015): ***Verdoia M****, Barbieri L, Schaffer A, Nardin M, Marino P, Suryapranata H, De Luca G. " Advanced age and high-residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor"*

ESC 2015 – POSTER PRESENTATION (August 2015): Barbieri L, ***Verdoia M****, Marino P, Suryapranata H, De Luca G. "Risk and benefits of triple therapy in patients undergoing percutaneous coronary stent implantation requiring chronic oral anticoagulation: a meta-analysis of 12 trials"*

ESC 2015 – POSTER PRESENTATION (August 2015): ***Verdoia M,*** *Nardin M, Barbier L, Schaffer A,Marino P, Suryapranata H,De Luca G. "Diabetes mellitus, glucose control parameters and platelet reactivity in ticagrelor treated patients"*

ESC 2015 – POSTER PRESENTATION (August 2015): ***Verdoia M****, Nardin M, Barbieri L, Sartori S, Schaffer A, Di Giovine G, Marino P, Suryapranata H, De Luca G. "Vitamin D levels and high--residual platelet reactivity in patients receiving dual antiplatelet therapy with clopidogrel or ticagrelor"*

ESC 2015 – POSTER PRESENTATION (August 2015): Di Giovine G, ***Verdoia M,*** *Schaffer A, Barbieri L, Marino P, Suryapranata H, De Luca G. "Impact of age on fibrinogen levels and its relationship with coronary artery disease: a single center study".*

Publication facts

1) ***Verdoia*** *M, Sartori C, Pergolini P, Nardin M, Rolla R, Barbieri L, Schaffer A, Marino P, Bellomo G, Suryapranata H, De Luca G. Immature platelet fraction and high-on treatment platelet reactivity with ticagrelor in patients with acute coronary syndromes. J Thromb Thrombolysis. 2015 Sep 14. [Epub ahead of print].*

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