Vitamin D receptor is expressed within human carotid plaques and correlates with pro-inflammatory M1 macrophages

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Abbreviation list

CV: Cardiovascular

VDR: Vitamin D receptor

CEA: Carotid endarterectomy

NASCET: North American Symptomatic Carotid Endarterectomy Trial

ECST: European Carotid Surgery Trial

ACST: Asymptomatic Carotid Surgery Trial

NYHA: New York Heart Association

MACE: Major Adverse Cardiovascular Event

CRP: C-Reactive Protein

MACE: Major Acute Cardiovascular Event

CCL: chemokine (CC motif) ligand

INF: Interferon

TLR: Toll-like Receptor

IQR: interquartile range

ROC: Receiver Operating Characteristic Non-contrast Cranial Computed Tomography

NIHSS: National Institutes of Health Stroke Scale

AUC: Area under the curve

CI: Confidence Interval

HR: Hazard ratio

SD: Standard Deviation

Abstract

The role of Vitamin D system in cardiovascular diseases remains controversial. Here, we investigated whether intraplaque levels of vitamin D receptor (VDR) predicted major adverse cardiovascular events (MACEs) at 18 month-follow-up and correlated with macrophage subsets in 164 patients undergoing endarterectomy for carotid stenosis. In human carotid plaque portions upstream and downstream the blood flow, VDR, lipid, collagen, as well as macrophage subsets were determined. Human primary monocytes were then differentiated *in vitro* to M1 and M2 macrophages and treated with 1,25(OH)₂D₃.

Intraplaque VDR positively correlated with total and M1 macrophages. According to the result of ROC curve analysis, downstream portions of plaques having high VDR expression were characterized by increased M1 macrophages. Kaplan-Meier analysis showed that the risk of MACEs was greater in patients having low downstream VDR levels (8.2% vs. 1.3%; $p=0.005$). Cox proportional hazard regression analyses confirmed that MACE risk decreased with increasing downstream VDR (adjusted HR 0.78 [95% CI 0.62-0.98]; $p=0.032$). *In vitro*, VDR expression was prevalent in M1, but not M2. Incubation of M1 macrophages with 1,25(OH)₂D₃, increased VDR expression and suppressed toll-like receptor 4 expression. These results suggest that low intraplaque VDR expression predict MACEs in patients with carotid stenosis potentially involving M1 macrophages.

Key words: Atherosclerosis, macrophage, major adverse cardiovascular events, vitamin D, vitamin D receptor.

1. Introduction

Vitamin D deficiency was initially supposed to be a condition increasing cardiovascular (CV) risk [1], because low circulating levels of 25(OH)D₃ (also known as vitamin D) have been associated with classical CV risk factors, including hypertension, dyslipidaemia, insulin resistance, and overweight/obesity [2-5]. However, in subsequent randomized clinical trials, vitamin D supplementation strategies failed to improve CV outcome [1]. Poor stratification for confounding factors, large variability of compounds used for supplementation and the lack of consensus definition of vitamin D deficiency may partially explain these discouraging clinical results.

Furthermore, from a pathophysiological point of view, many studies support an important role of the vitamin D in the regulation of critical pathways involved in vascular physiology, such as the renin-angiotensin-aldosterone system [6], and fibroblast growth factor 23/klotho axis [7] and the major influence of autocrine/paracrine-related properties of vitamin D. The discovery of extra-renal expression of CYP27B1 (the rate-limiting enzyme catalysing hydroxylation of 25(OH)D₃ to the active form 1,25(OH)₂D₃ and the ubiquitous distribution of vitamin D receptor (VDR) further reinforced the importance of a local control of vitamin D signalling in modulating vascular pathologies. Several cell types involved in atherogenesis (i.e., vascular smooth muscle cells, endothelial cells, macrophages, and dendritic cells) showed a CYP27B1 activity and a VDR expression *in vitro*, highlighting the importance of an autocrine/paracrine control of vitamin D signalling [1]. However, these local effects of vitamin D were marginally investigated within human atherosclerotic plaques and resident inflammatory cells, such as macrophages. Furthermore, the potential role of local vitamin D on toll-like receptor expression (known to be relevant in atherogenesis) [8] on different macrophage subsets remains elusive.

In this study, we assessed the prognostic value of the intraplaque VDR expression in upstream and downstream portions of carotid atherosclerotic plaques. In addition, we investigated the potential association between levels of VDR and the different subtype of macrophages. Finally, both function and membrane expression of VDR were assessed *in vitro* on different macrophage subsets (i.e. M1 and M2).

2. Methods

2.1 Patients and clinical assessment

From March 2008 to June 2011, we consecutively enrolled 201 patients with extra cranial high-grade internal carotid stenosis (>70% luminal narrowing) [9] in an observational cohort at a single center (IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy). Some of the cohort samples have already been used for analysis and published [10]. Among the total cohort (n=201), 37 carotid endarterectomy were missing, leaving 164 samples available for analyses that were included in the present sub-study. As previously described [11], all patients underwent elective carotid endarterectomy (CEA) according to the recommendations published by the North American Symptomatic Carotid Endarterectomy Trial (NASCET) [12], the European Carotid Surgery Trial (ECST) [13], and the Asymptomatic Carotid Surgery trial (ACST) [14]. The Ethics Committee of IRCCS Azienda Ospedaliera Universitaria San Martino-IST Istituto Nazionale per la Ricerca sul Cancro in Genoa, Italy approved this protocol, performed in accordance to the guidelines of the Declaration of Helsinki. Patients gave informed consent before entering in the study. The day prior to CEA, serum samples were obtained to measure circulating markers of cardiovascular vulnerability. Medications reported in Table 1 were not modified in the 2 months prior to enrolment. Exclusion criteria were: spontaneous cerebral embolism up to 30 minutes preoperatively or during the dissection phase of the operation, malignant

hypertension, acute coronary artery disease, any cardiac arrhythmias, congestive heart failure (II, III, and IV New York Heart Association [NYHA] classes), liver or renal disorder or function abnormalities, acute and chronic infectious diseases, autoimmune and rheumatic diseases, cancer, endocrine diseases, inflammatory bowel diseases and anti-inflammatory (other than aspirin) medications, oral anticoagulant treatments, and hormone, cytokine or growth factor therapies.

2.2 Study endpoints

We performed a pilot study to investigate human intraplaque VDR expression levels. The primary end-point of the study was to determine if VDR intraplaque expression levels at time of CEA could predict the occurrence of major adverse cardiovascular events (MACEs, defined as fatal or non-fatal acute coronary syndrome and stroke) over a follow-up of 18 months [10]. The secondary end-points consisting in determining the potential correlations between circulating vitamin D levels or intraplaque VDR with histological parameters of inflammation in upstream and downstream portions of carotid plaques. Two independent investigators who were blinded to the biochemical and histological analyses adjudicated the study endpoints. Information was obtained during a check-up visit at 18 months and was further confirmed by checking patients' medical file, targeting medical history relevant to the study endpoint, as noted in their medical file.

2.3 Detection of biochemical and inflammatory biomarkers

Routine auto-analysers were used to assay haematological parameters and blood chemistry including total cholesterol, low- and high-density lipoprotein cholesterol, triglycerides fibrinogen and glycaemia. Total 25(OH) vitamin D₃ measurements were performed at the Division of Laboratory Medicine of the Geneva University Hospitals on a Cobas 6000

immunoanalyzer (Roche Diagnostics, Rotkreuz, Switzerland) using Elecsys Vitamin D Total reagents (Roche Diagnostics). Inter-assay coefficients of variation (n=68) of the method were 8.0% at 47.9 nmol/l and 6.3% at 108.7 nmol/l. Serum levels of C-reactive protein (CRP) and the CC chemokines CCL2, CCL3, CCL4, CCL5 (all from R&D systems, Minneapolis, MN) were measured by colorimetric enzyme-linked immunosorbent assay (ELISA) following the manufacturer's instructions. The limits of detection were 31.25 pg/ml for CRP, 15.60 pg/ml for CCL2, 7.81 pg/ml for CCL3, 15.60 pg/ml for CCL4, 15.60 pg/ml for CCL5. Mean intra- and inter-assay coefficients of variation were <8% for all markers measured by ELISA.

2.4 Endarterectomy specimen processing

As previously reported [11], the internal carotid plaque specimens were cut right after surgical excision, perpendicularly to the long axis through the point of maximum stenosis to obtain two portions (upstream and downstream of the blood flow). Each portion was further divided perpendicularly to the long axis in the middle into two sub-segments. Upstream and downstream carotid specimens were then frozen in cryoembedding medium for histological analysis and serially cut in 7- μ m sections.

2.5 Oil Red O staining for lipid content

Eight sections per each portion (upstream and downstream the blood flow) separated by 105 μ m from each other were stained and counterstained with Mayer hemalune's solution, as previously described [11, 15]. Quantifications were performed with MetaMorph™ 6 software. Data were calculated as ratios of stained area on total lesion area.

2.6 Sirius red staining for collagen content

Eight section per each portion (upstream and downstream the blood flow) separated by 105 μm from each other were stained as previously described [11, 15]. The sections were photographed under light microscopy (in order to evaluate total collagen content) and under polarization microscopy. Total collagen content was quantified by MetaMorph™ 6 software. Results on total collagen were presented as percentages of stained area on total lesion area.

2.7 Immunostaining of endarterectomy specimens

Eight sections per each portion separated by 105 μm from each other were fixed in acetone at room temperature and immunostained with specific antibodies. For detecting VDR, we used anti-human VDR (dilution: 1:500; Abcam, UK). For detecting macrophages, we used anti-human CD68 (markers of total macrophages, diluted: 1:100; Dako Corporation, CA), anti-human CD86 (marker of M1 macrophages, diluted: 1:100; GeneTex Inc., Irvine, CA), anti-human HLA-DR (marker of M1 macrophages, diluted 1:100; Dako Corporation), and anti-human CD163 (marker of M2 macrophages, diluted: 1:50; AbD Serotec, Oxford, UK) [16]. Quantifications were performed using MetaMorph™ 6 software. Data were presented as percentages of stained area on total lesion area.

2.8 Monocyte purification

Monocytes were isolated from blood buffy coats of healthy volunteers provided by the Geneva Hospital blood transfusion center, as previously described [17]. Monocyte purity was routinely controlled by flow cytometry and the isolated cell population consisted of CD14⁺ cells (>90%), CD3⁺ cells (<1%), and CD19⁺ cells (<1%). Cells were cultured in RPMI containing 10% FBS (Gibco BRL-Life Technologies, Grand Island, NY) [18].

2.9 Mono-macrophage polarization and treatment

Mono-macrophages were respectively polarized toward M1 or M2 subsets either by 24h incubation with 500 U/ml of interferon(INF)-gamma (Roche Diagnostic, Mannheim, Germany) or 20 ng/ml of interleukin (IL)-4 (PeproTech, Inc, London, UK) (16). When indicated, 100 nmol/l of vitamin 1,25(OH)₂D₃ (Sigma-Aldrich Chemie GmbH, Germany) were added in the medium for 24h or 48h [19].

2.10 Flow Cytometry analysis

VDR and toll-like receptor (TLR)-4 expression were determined on non-polarized (M0) or polarized (M1 or M2) macrophages after treatment with INF-gamma or IL-4 for 24h (day 1). In addition, VDR and TLR4 expression were determined after additional treatment with 100 nmol/l 1,25(OH)₂D₃ for 24h or 48h (day 2 and day 3, respectively). For VDR staining, 5x10⁵ cells per condition were fixed with 4% of PAF for 10 min, then permeabilized with 0.1% saponin in PBS-2% BSA and incubated with mouse monoclonal anti-CD32 and anti-CD64 antibodies (0.5µg/5x10⁵ cells) (Biolegend) for 10 min. Subsequently, of 0.5 µg of rabbit polyclonal anti-human VDR (ab3508, Abcam) were added for 30 min. After washing, cells were incubated with 4 µg/ml of AF488-conjugated goat anti-rabbit polyclonal antibodies (Invitrogen Co., Carlsbad, CA) for 30 min. For TLR4 staining, 5x10⁵ cells per condition were incubated with the mix of mouse monoclonal anti-CD32 and anti-CD64 antibodies in PBS-2% BSA for 10 min on ice, after which 8 µg/ml of APC-mouse monoclonal anti-TLR4 antibodies (HTA125, Biolegend, San Diego, CA) were added for 30 min. Staining was quantified with ACCURI C6 flow cytometer (BD Biosciences, San Jose, CA) and analysed using FlowJo data analysis software (version 10.0.7) (FlowJo LLC, Ashland, OR).

2.11 Statistical analysis

Analyses were performed with IBM SPSS Statistics for Windows, Version 21.0 (IBM CO., Armonk, NY), and STATISTICA 12 (StatSoft, Inc. Tulsa, OK). In the clinical study, categorical data are presented as relative and absolute frequencies, whereas continuous variables were expressed median and interquartile range (IQR). Intergroup comparisons were drawn by Fisher's exact test and Mann-Whitney *U*-test, as appropriate. Ranked Spearman correlation coefficients were performed to establish correlations between serum and intraplaque biomarkers in both upstream and downstream portions of atherosclerotic plaque. The prognostic ability of VDR was assessed in a post-hoc manner based upon receiver operator characteristic (ROC) curve, using MedCalc 12.5 (MedCalc Software, Ostend, Belgium). The area under the curve (AUC) was given with 95% confidence interval (CI) and the cut-off point of VDR was calculated maximizing the sensitivity in accordance to the Youden's index. Kaplan-Meier survival analysis with Log Rank test was performed to estimate cumulative event rate during 18 months after CEA and to calculate the corresponding risk difference according to categorized VDR. Finally, the effect of VDR expression on MACE risk was estimated by Cox proportional hazards models and expressed with hazard ratios (HR) and 95% CI. In the multivariate model, we adjusted for age, gender and serum levels of 25(OH)D₃. In the basic research study, the data were presented as mean $\pm$ standard deviation (SD). Statistical analyses were performed using Mann-Whitney *U*-test. For all statistical analyses a 2-sided *p*-value <0.05 was considered as statistically significant.

3. Results

3.1 Patient characteristics

Clinical and biochemical characteristics of the study cohort, including medications, are listed in Table 1. Median patient age was 72 (67-77) years, with a high prevalence of males (66.5%) and hypertensive patients (71.9%). Biochemical parameters, haematological are shown in

Table 1, whereas circulating levels of vitamin 25(OH)D₃, CRP and CC chemokines are reported in Table 2. These results indicate that the cohort of patients was not affected by relevant acute inflammation.

3.2 Circulating vitamin 25(OH)D₃ and intraplaque VDR levels correlate with intraplaque inflammatory parameters

A significant correlation between serum levels of vitamin 25(OH)D₃ and intraplaque lipid content ($r=0.242$; $p=0.003$) was observed in the upstream portions of the plaques (Table 3). With regard to the downstream portion, serum vitamin 25(OH)D₃ showed a positive correlation with VDR ($r=0.166$; $p=0.038$) and total macrophages ($r=0.177$; $p=0.027$) (Table 3). As reported in Table 4, we also analysed the relationship between intraplaque VDR levels and different intraplaque parameters of inflammation. In the upstream portions, VDR directly correlated with the M1 macrophage markers CD86 ($r=0.371$; $p<0.001$) and HLA-DR ($r=0.402$; $p<0.001$) (Table 4 and Figure 1). Similarly, in the downstream portions, VDR directly correlated with total macrophages ($r=0.365$; $p<0.001$) and the M1 markers CD86 ($r=0.279$; $p<0.001$) and HLA-DR ($r=0.304$; $p<0.001$) (Table 4 and Figure 2). In both upstream and downstream portions of carotid plaques, positive areas for VDR corresponded to intraplaque zones positive for M1 macrophages markers (Figures 1 and 2). Conversely, no significant correlation was found between VDR and lipids, collagen and M2 macrophages (CD163⁺ cells) in both upstream and downstream portion of the plaque (Figures 1 and 2).

3.3 VDR levels in downstream portion of carotid plaques predict future MACEs

ROC curve analysis showed that upstream expression of VDR failed to predict the occurrence of MACEs (Figure 3A). Conversely, downstream expression of VDR had significant prognostic accuracy to predict subsequent MACEs (AUC 0.718 [95% CI 0.642-0.785];

$p=0.001$) (Figure 3B). In accordance with the Youden Index, a downstream VDR expression 7.13% was identified as the best cut-off point, having a sensitivity of 100% and a specificity of 49.03%. The downstream plaques were then categorized by low ($\leq 7.13\%$) or high ($> 7.13\%$) VDR levels. At this cut-off, Kaplan-Meier analysis indicated that patients with low VDR expression had a higher rate of MACEs than patients expressing high VDR levels (Figure 4). Of the 9 MACEs observed during 18 months of follow-up, 8 occurred in the group with low VDR expression, whereas only 1 was observed in patients with VDR values above the cut-off (8.2% vs. 1.3%; $p<0.001$) (Figure 4). Cox proportional hazard regression analyses further confirmed these findings. The risk of MACEs decreased with increasing VDR expression in the downstream portion of the plaque (HR 0.82 [95% CI 0.67-0.99]; $p=0.046$) (Table 5). These results remained statistically significant also after adjustment for age, gender, and serum levels of vitamin 25(OH)D₃ (HR 0.78 [95% CI 0.62-0.98]; $p=0.032$) (Table 5).

3.4 *VDR levels in downstream portions of carotid plaques are associated with intraplaque content of M1 macrophages*

The amount of different macrophages subsets was quantified according to low versus high VDR expression in downstream regions of plaques. In the upstream portions, intraplaque content of macrophages did not differ between groups, even when analysing the M1 and M2 subsets separately (Figure 5A-D). Conversely, downstream portions with high VDR levels were characterized by an increased M1 macrophage content ($p=0.001$) (Figure 5A). Specifically, high M1 macrophages were found in plaques with high VDR levels, as indicated by the high expression of CD86 ($p=0.002$) and HLA-DR ($p=0.010$) (Figure 5B and C). No difference for M2 macrophages were observed (Figure 5D).

3.5 *VDR is highly expressed on M1 macrophages and modulates the surface expression of toll-like receptor 4*

To further investigate the expression and activity of VDR on human macrophage subsets, we performed an *in vitro* analysis of VDR expression on the M1 and M2 macrophage subtype after differentiation. As compared to undifferentiated macrophages (M0), M1 (but not M2) macrophages showed a significant and persistent VDR expression at 24, 48 and 72 hours (Figure 6A). Incubation with $1,25(\text{OH})_2\text{D}_3$ further up-regulated VDR expression on M1 macrophages (Figure 6B). We also observed that M1 macrophages expressed high levels of TLR4, as compared with M2 (Figure 6C). Incubation with vitamin $1,25(\text{OH})_2\text{D}_3$ abrogated TLR4 expression on M1 macrophages, with a significant effect observed at 48 hours (Figure 6D).

4. Discussion

The novel finding of this study resides in the fact that circulating levels of vitamin D were not predictive of subsequent MACE, whereas low VDR expression within CEA biopsies was. These results extend previous negative finding regarding the possible CV prognostic value of circulating vitamin D levels and further emphasizes the importance of local vitamin D expression in atherogenesis and its related complications in humans. These results are in accordance with previous *in vivo* results derived from monkey's models, which demonstrated an inverse relationship between VDR and atherosclerotic the plaque size [20]. However, the authors did not clarify which cells expressed VDR and whether low VDR expression was a result of plaque progression and if it contributed directly to atherosclerotic plaque vulnerability. Furthermore, in this study we analysed separately upstream and downstream portions of carotid plaques allowing us to evaluate the effect of shear stress on intraplaque macrophages polarization. We retrieved a positive correlation between intraplaque VDR and

total macrophages in the downstream portions of the plaques. In addition, we observed that intraplaque VDR was positively correlated with CD86 and HLA-DR, two hallmarks of M1 macrophages [16]. Despite highly speculative, we believe that both different hemodynamic forces and cell recruitment/activation might influence expression of VDR as well as other receptors on M1 macrophages in human carotid plaques [21, 22]. However, since M1 macrophages are traditionally considered as a pro-inflammatory subset associated with plaque vulnerability [16], this association with VDR expression is rather intriguing as the opposite observation would have been expected. Indeed the activation of vitamin D signalling was shown to reverse pro-atherogenic cholesterol metabolism in macrophages by downregulating the expression of scavenger receptors (SR-A1 and CD36), TLRs [23, 24] and promoting cholesterol efflux [25-27]. Further contrasting with current results, vitamin 1,25(OH)₂D₃ was shown to promote the M1 switching to the anti-inflammatory M2 phenotype [19, 28]. Nevertheless, the fact that vitamin D up-regulated VDR expression on M1 macrophages could be interpreted as a negative feedback loop mechanism to contain the pro-inflammatory M1 response. The fact that vitamin D treatment decreased TLR4 expression on M1 macrophages, and that low VDR expression in the downstream part of atherosclerotic plaque acted as an independent predictor of MACE during follow-up is in line with this hypothesis. These results suggested an autocrine control of vitamin D with potential role as feedback mechanism with inhibitory effect on macrophage activity [29, 30], through the modulation of TLR4 expression. In this context, the down-regulation of TLR4 might represent a mechanism of switching from M1 to M2 macrophages as previously suggested [31], but this intriguing hypothesis requires further investigations. In addition, whether this autocrine control of VDR could also modulate different Vitamin D enzymes, including the hydroxylases CYP27A1, CYP27B1, and CYP24A [1], remains unknown.

This study has many limitations. As first, the lack of a full description of the intraplaque vitamin D system (including enzymes) that should be addressed in future researches. Secondly, knowing whether portion of atherosclerotic plaque with low VDR had a higher TLR4 expression would have reinforced the present *in vitro* results. Nevertheless, due to the limited amount of available material, we could not assess TLR expression in human atherosclerotic plaques. Thirdly, the relative small sample size and the involvement of a single center for patient recruitment may represent a further limitation in terms of possible selection bias. Finally, the post-hoc definition of the VDR cut-off point by ROC curve prompts us to prospectively validate the defined cut-off in another larger prospective cohort.

In conclusion, the present study demonstrated that low VDR levels in downstream portions of carotid plaques predicted MACEs, but not the plasmatic levels of vitamin D. In addition, intraplaque VDR levels were positively associated with M1 macrophage content. *In vitro*, incubation with 1,25(OH)₂D₃ on differentiated M1 macrophages increased VDR and decreased TLR4 expression on cell surface membranes, suggesting an anti-inflammatory effect of Vitamin D on this cell subset. Although not conclusive, our results strongly indicate VDR as an active anti-inflammatory mediator within atherosclerotic plaques.

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Figure Legends

Figure 1. Representative microphotographs of the upstream portion of human carotid atherosclerotic plaques. Immunostainings for total lipid content (Oil Red O), vitamin D receptor (VDR), total macrophage infiltrate (M ϕ ; CD68⁺ cells), M1 macrophages (CD86⁺ and HLA-DR⁺ cells), and M2 macrophages (CD163⁺ cells) were performed. The arrows and high magnifications pictures show that VDR and M1 macrophage subset localize in the same regions of atherosclerotic plaques.

Figure 2. Representative microphotographs of the downstream portions of carotid plaques. Immunostainings for total lipid content (Oil Red O), vitamin D receptor (VDR), total macrophage infiltrate (M ϕ ; CD68⁺ cells), M1 macrophages (CD86⁺ and HLA-DR⁺ cells), and M2 macrophages (CD163⁺ cells) were performed. The arrows and high magnifications pictures show that VDR and M1 macrophage subset localized in the same regions of atherosclerotic plaques.

Figure 3. Receiver operator characteristic (ROC) curve analysis for VDR expression in upstream and downstream portions of carotid plaques. The predictive value of VDR expression toward the occurrence of major adverse cardiovascular events (MACEs) at 18-month follow-up was tested. **A.** VDR expression in upstream portions. **B.** VDR expression in downstream portions.

Figure 4. Low downstream expression of VDR is associated with increased rate of major adverse cardiovascular events (MACEs) at 18 month-follow up. Kaplan-Meier curve according to low (VDR -: $\leq 7.13\%$) and high (VDR +: $> 7.13\%$) VDR expression levels in downstream regions.

Figure 5. Low expression of VDR in downstream portions of carotid plaques is associated with an increase in M1 macrophage content. Macrophage content in both up and downstream portions of atherosclerotic plaques was analysed considering low (VDR -: $\leq 7.13\%$) and high (VDR +: $> 7.13\%$) VDR expression levels in downstream regions. **A.** Total macrophage (CD68+ area) content. **B.** M1 macrophage (CD86+ area) content. **C.** M1 macrophage (HLA-DR+ area) content. **D.** M2 macrophage (CD163+ area) content.

Figure 6. Treatment with vitamin D *in vitro* upregulates VDR and downregulates TLR4 expression on M1 macrophages. VDR and TLR4 expression was assessed on M1 and M2 macrophages *in vitro* in the presence or absence of 100 nmol/l of 1,25(OH)₂D₃ (the active form of vitamin D). **A.** VDR expression on M1 and M2 macrophages up to 3 days of culture. **B.** VDR expression on M1 macrophages exposed to 1,25(OH)₂D₃ or control medium. **C.** TLR4 expression on M1 and M2 macrophages up to 3 days of culture. **D.** TLR4 expression on M1 macrophages exposed to 1,25(OH)₂D₃ or control medium.

Figure 1

Upstream

Figure 2

Downstream

Figure 3

A

B

Figure 4

Figure 5

Figure 6

Table 1. Clinical characteristics of overall cohort at admission.

Overall cohort (n=164)	
Demographic	
Age, yr. (IQR)	72 (67-77)
Males, no. (%)	109 (66.5)
Symptomatic, no. (%)	34 (20.73)
Systolic BP*, mmHg (IQR)	140 (130-145)
Diastolic BP, mmHg (IQR)	80 (80-90)
Waist circumference (cm)	90 (87-97)
Carotid stenosis (%)	80 (75-90)
Hypertension, no. (%)	118 (71.9)
Active smokers, no. (%)	42 (25.6)
Previous smokers, no. (%)	73 (44.5)
Type 2 diabetes, no. (%)	31 (18.3)
Dyslipidaemia, no. (%)	99 (60.4)
Chronic CAD†, no. (%)	28 (17.1)
Medications	
RAAS‡ inhibitors, no. (%)	86 (49.4)
ACE-I§, no. (%)	10 (6.1)
ARBs , no. (%)	76 (43.3)
β-blockers, no. (%)	48 (29.3)
Calcium antagonists, no. (%)	48 (29.3)
Diuretics, no. (%)	19 (11.6)
Statins, no. (%)	86 (52.4)
Antiaggregants, no. (%)	132 (80.4)
Aspirin, no. (%)	96 (58.5)
Thienopyridine, no. (%)	36 (21.9)
Anticoagulants (heparin)	9 (5.5)
Oral antidiabetics, no. (%)	23 (14.0)
Insulin, no. (%)	4 (2.4)
Laboratory findings	
Haematology	
Total WBC#, no. x 10 ⁹ /l (IQR)	7.23 (6.26-8.35)
Neutrophil, no. x 10 ⁹ /l (IQR)	4.64(3.65-5.53)
Lymphocyte, no. x 10 ⁹ /l (IQR)	1.78 (1.42-2.15)
Monocyte, no. x 10 ⁹ /l (IQR)	0.45 (0.37-0.57)
Platelet, no. x 10 ⁹ /l (IQR)	232 (192-280)
Red blood cell, no. x10 ¹² /l (IQR)	4.70 (4.40-4.95)
Chemistry	
Serum total-c** mg/dl (IQR)	194 (164-219)
Serum LDL-c†† mg/dl (IQR)	116 (89-143)
Serum HDL-c‡‡ mg/dl (IQR)	49 (42-61)
Serum TAG§§ mg/dl (IQR)	120 (92-162)
Fibrinogen, mg/dl (IQR)	3.69 (3.13-4.21)
Fasting glycaemia, mg/dl (IQR)	101 (91-115)

Data are expressed as median (interquartile range [IQR]) or number [no.] (percentages [%]).

* BP: blood pressure

† CAD: coronary artery disease

‡ RAAS: renin-angiotensin-aldosterone system.

§ ACE-I: angiotensin converting enzyme inhibitor

|| ARBs: angiotensin receptor blockers

WBC: white blood cells

** total-c: total cholesterol

†† LDL: low-density lipoprotein

‡‡ HDL: high-density lipoprotein

§§ TAG: triglyceride

Table 2. Serum levels of vitamin D, hs-CRP and CC chemokines.

	Overall cohort (n=164)
25(OH)D ₃ [*] , nmol/l (IQR)	32.03 (21.31-50.40)
CRP [†] , µg/ml (IQR)	2.34 (0.87-4.99)
CCL2 [‡] , pg/ml (IQR)	65.29 (15.63-148.05)
CCL3, pg/ml (IQR)	8.24 (7.80-12.18)
CCL4, pg/ml (IQR)	29.47 (15.62-47.32)
CCL5, ng/ml (IQR)	54.23 (33.3-91.01)

Data are expressed as median (interquartile range [IQR]).

* 25(OH)D₃: 25-hydroxyvitamin D

† hsCRP: C-reactive protein

‡ CCL: chemokine (C-C motif) ligand

Table 3. Relationship between serum levels of 25-hydroxyvitamin D and intraplaque biomarkers.

	25(OH)D₃[*], nmol/l	
	<i>r</i>	<i>p</i> -value
Upstream portion		
VDR [†] , %	-0.034	0.685
Oil Red O, %	0.242	0.003
Total collagen, %	0.044	0.594
Total macrophages, %	0.141	0.086
M1 subset (CD86 ⁺) , %	-0.008	0.919
M1 subset (HLA ⁺) [§] , %	0.101	0.233
M2 subset (CD163 ⁺), %	-0.021	0.805
Downstream portion		
VDR, %	0.166	0.038
Oil Red O, %	0.010	0.900
Total collagen, %	0.106	0.190
Total macrophages, %	0.177	0.027
M1 subset (CD86 ⁺), %	0.083	0.308
M1 subset (HLA ⁺), %	-0.133	0.101
M2 subset (CD163 ⁺), %	-0.030	0.709

Correlations were performed by Spearman's rank correlation coefficient.

* 25(OH)D₃: 25-hydroxyvitamin D

†VDR: vitamin D receptor

|| CD: cluster of differentiation

§ HLA: human leukocyte antigen

Table 4. Correlation between vitamin D receptor expression and intraplaque biomarkers.

Upstream		
VDR[*], % vs.	<i>r</i>	<i>p</i> -value
Oil Red O, %	0.016	0.621
Total collagen, %	-0.105	0.482
Total macrophages, %	0.065	0.408
M1 subset (CD86 ⁺) [†] , %	0.371	<0.001
M1 subset (HLA ⁺) , %	0.402	<0.001
M2 subset (CD163 ⁺), %	0.148	0.072
Downstream		
VDR, % vs.	<i>r</i>	<i>p</i> -value
Oil Red O, %	-0.010	0.899
Total collagen, %	0.048	0.541
Total macrophages, %	0.365	<0.001
M1 subset (CD86 ⁺), %	0.279	<0.001
M1 subset (HLA ⁺), %	0.304	<0.001
M2 subset (CD163 ⁺), %	-0.005	0.947

Correlations were performed by Spearman's rank correlation coefficient.

* VDR: vitamin D receptor

† CD: cluster of differentiation

|| HLA: human leukocyte antigen

Table 5. Cox hazard proportional model showing the predictive value of categorized downstream VDR expression (cutoff $\leq 7.13\%$) towards MACE occurrence during 18 months of follow-up.

	Univariate model			Multivariate model		
	HR [*]	95% CI [†]	<i>p</i> -value	HR	95% CI	<i>p</i> -value
MACEs[‡]						
Downstream VDR	0.82	0.67-0.99	0.046	0.78	0.62-0.98	0.032
Age	0.96	0.89-1.04	0.382	0.95	0.87-1.04	0.271
Gender, male	0.97	0.24-3.88	0.966	0.61	0.14-2.68	0.514
Serum vitamin D	1.01	0.98-1.02	0.859	1.01	0.98-1.02	0.628

* HR: hazards ratio

† CI: confidence interval

‡ MACEs: major adverse cardiovascular events

|| VDR: vitamin D receptor