

RESEARCH ARTICLE

Vitamin D Status in Rheumatoid Arthritis: Inflammation, Arterial Stiffness and Circulating Progenitor Cell Number

Alberto Lo Gullo¹*, Giuseppe Mandraffino¹*, Gianluca Bagnato¹, Caterina Oriana Aragona¹, Egidio Imbalzano¹, Angela D'Ascola², Francesco Rotondo³, Antonella Cinquegrani⁴, Enricomaria Mormina⁴, Carlo Saitta¹, Antonio Giovanni Versace¹, Maria Adriana Sardo¹, Renato Lo Gullo¹, Saverio Loddo², Antonino Saitta¹

1 Department of Clinical and Experimental Medicine, University of Messina, Messina, Italy, **2** Department of Biochemical, Physiological and Nutritional Sciences, University of Messina, Messina, Italy, **3** Department of General Surgery and Oncology, University of Messina, Messina, Italy, **4** Department of Biomedical Sciences and of Morphologic and Functional Images, University of Messina, Messina, Italy

* These authors contributed equally to this work.

* gmandraffino@unime.it



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Abstract

Background and Aims

Suboptimal vitamin D status was recently acknowledged as an independent predictor of cardiovascular diseases and all-cause mortality in several clinical settings, and its serum levels are commonly reduced in Rheumatoid Arthritis (RA). Patients affected by RA present accelerated atherosclerosis and increased cardiovascular morbidity and mortality with respect to the general population. In RA, it has been reported an impairment of the number and the activity of circulating proangiogenic haematopoietic cells (PHCs), including CD34+, that may play a role in endothelial homeostasis. The purpose of the study is to investigate the association between vitamin D levels and PHCs, inflammatory markers, and arterial stiffening in patients with RA.

Methods and Results

CD34+ cells were isolated from 27 RA patients and 41 controls. Vitamin D levels, C-reactive protein (CRP), fibrinogen, pulse wave velocity (PWV), and carotid intima-media thickness (cIMT) were also evaluated. CD34+ count and vitamin D levels were lower in RA patients as compared to controls, while fibrinogen, CRP, PWV and cIMT were higher in RA patients. CD34+ cell number appeared to be associated with vitamin D levels, and negatively correlated to fibrinogen and early atherosclerosis markers (PWV and cIMT); vitamin D levels appear also to be inversely associated to fibrinogen.

Conclusions

RA patients with moderate disease activity presented with low vitamin D levels, low CD34+ cell count, increased PWV and cIMT; we found that vitamin D deficiency is associated to

CD34+ cell reduction in peripheral blood, and with fibrinogen levels. This suggests that vitamin D might contribute to endothelial homeostasis in patients with RA.

Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease associated with increased morbidity and mortality, mainly due to cardiovascular events [1]. The mechanisms behind the higher prevalence of cardiovascular disease (CVD) in RA is not fully understood, but it is likely that the incidence may be linked to vascular inflammation and consequently to accelerated atherosclerosis [2, 3]. Atherosclerosis and RA share many common inflammatory mediators, and the mechanisms leading to synovial inflammation are similar to those found in unstable atherosclerotic plaque; levels of inflammatory molecules, such as CRP, fibrinogen and cytokines, including interleukins (IL-s), may be altered in RA and promote proatherogenic activation and endothelial dysfunction, but also associate with other CV risk factors, such as changes in lipid levels, insulin resistance and oxidative stress that further contribute to vascular damage [2–4]. Circulating proangiogenic hematopoietic cells (PHCs) are a heterogeneous population of cells in different states of maturation with the ability to differentiate into a broad range of cell types of different organs and systems, including cardiomyocytes, smooth muscle cells, and endothelial progenitor cells, as well as hematopoietic, stromal, and epithelial cells [5–7]. However, PHCs have been shown to contribute to postnatal vasculogenesis and they may participate in the turnover of healthy and damaged endothelium, delaying the development of atherosclerosis and CVD [6]. PHCs are negatively affected by risk factors for CVD and positively by changes in lifestyle, are associated with life expectancy, and their number is considered an independent predictor of CVD and all causes mortality [6, 8, 9].

Many different surface antigen, often co-expressed by endothelial and hematopoietic cells, have been already proposed to identify putative endothelial progenitors cells (EPCs), including CD34, CD117, CD133, CD105, CD144, CD184, CD309 (KDR or VEGFR2), acetylated low density lipoprotein, and various plant lectins [7]; however, the question of which cell phenotype better identifies the “true” circulating EPC remains unsolved, since the more widely studied PHC phenotypes do not give rise to mature ECs and are different from endothelial forming colony cells [10, 11]. Despite consistent evidence of CD34 expression by many different cell types, there is still a misconception that this surface antigen identifies a cell of hematopoietic origin, and experimentally, CD34+ cells are often regarded as hematopoietic contamination and subsequently disregarded [6]. Although the mechanisms on how CD34+ cells exert their role in angiogenesis are uncertain CD34 appears to identify a cluster of cell types with progenitor and stem activity, and in many cases, the CD34+ population showed a more potent or pronounced differentiation capacity, and also transdifferentiation ability [6]. In addition, it was suggested that mature endothelial cells may de-differentiate and re-establish an overlapping endothelial-hematopoietic phenotype, including CD45 expression, also in adulthood; thus, even the expression of CD45, which is generally considered a specific pan-leukocyte marker, might not be a reliable watershed between the hematopoietic and endothelial lineage [12, 13]. Fadini and coll. evaluated the impact of different immunophenotypes of CPCs on the ability to predict or associate with CV risk factors and outcomes. They suggested that CD34+ cell count is closely linked to CV risk, better than CD133+ cell number and multiple positive phenotypes (CD34+/CD133+, CD34+/KDR+, CD133+/KDR+, and triple positive CD34+/CD133+/KDR+), and an extensive antigenic characterization of circulating CD34+ cells may not be useful for

this purpose [14, 15]. Consistently, CD34+ cell count has been suggested for CVD risk estimation in the clinical practice, rather than more complex EPC phenotyping, since the quantification of CD34+ cells, although not-specific for endothelial/CV repair, is already performed in most hematology laboratories in a standardized fashion [5, 6, 12]. Furthermore, a recent article indicated that CD34 hybrid cells promote endothelial colony-forming cell bioactivity and have a therapeutic potential for ischemic diseases [16]. Indeed, although it has been suggested that in RA patients the impairment of endothelial repair mechanisms may be at least in part due to the altered number or function of PHCs, many other cell types may cooperate in maintaining endothelial homeostasis, including the so-called angiogenic T cells (Tang), a subset of T cells recently described; Tang, in fact, seems to cooperate with circulating progenitors and enhance endothelial repair function, possibly through the secretion of proangiogenic cytokines [16–18].

In RA, PHCs are recruited into synovia and may participate in local angiogenesis [19]; the consequence of PHCs migration to the inflamed synovia would be paralleled by a reduction in the periphery [20] potentially impairing endothelial repair and leading to vascular damage. However, additional factors could be involved in modulating the number and activity of progenitor cells in RA [21, 22].

Adequate vitamin D status is important for optimal function of many organs and tissues throughout the body, including the cardiovascular system [23]. In several studies Vitamin D deficiency has been associated with atherosclerosis and cardiometabolic risk factors in non-RA populations, such as metabolic syndrome, hypertension, insulin resistance and CVD, as arterial dysfunction, arterial stiffness (AS), and atherosclerotic coronary disease [23–26]. In RA, low levels of vitamin D are noted to be common [27] and are described to be associated with different cardiometabolic risk factors [28].

Recently, vitamin D receptor has been detected in PHCs [29]. Furthermore, it has been described that vitamin D3 promotes cell proliferation of endothelial colony-forming cells by increasing vascular endothelial growth factor (VEGF) expression and pro-matrix metalloproteinases (MMP) activity [30]. Those data support the hypothesis that vitamin D might have a role in endothelium homeostasis.

The relationships between vitamin D, inflammatory markers, AS and PHCs in RA patients have not to date yet been investigated. An observational cross sectional study was designed to investigate the association between CD34+ cell number, vitamin D levels, and systemic inflammation. Pulse wave velocity and carotid intima-media thickness (cIMT), both patterns of pre-clinical atherosclerosis, were also evaluated in relation to inflammatory markers, CD34+ cell number, and vitamin D levels.

Materials and Methods

Subjects

Between October 2012 and May 2013, 563 outpatients were examined for the first time at the Rheumatology Division of the University of Messina and were referred for a clinical and instrumental screening; according to inclusion/exclusion criteria, only 27 subjects (8 men and 19 women) were considered eligible for this study (Fig 1): to be recruited for the study, subjects needed to be newly diagnosed, untreated, not have additional risk factors for atherosclerosis or CVD, and meet the retrospective application of the 1987 revised RA criteria of the American Rheumatism Association [31]; additionally, they should never have been treated with immunosuppressive drugs, long term corticosteroids and/or NSAIDs nor DMARDs. Patients previously treated with NSAIDs should not take drugs for at least two weeks before inclusion. To select a homogeneous population, only patients with “moderate” disease activity were enrolled, according to DAS28 value; DAS28 was calculated using CRP and the Nijmegen formula:

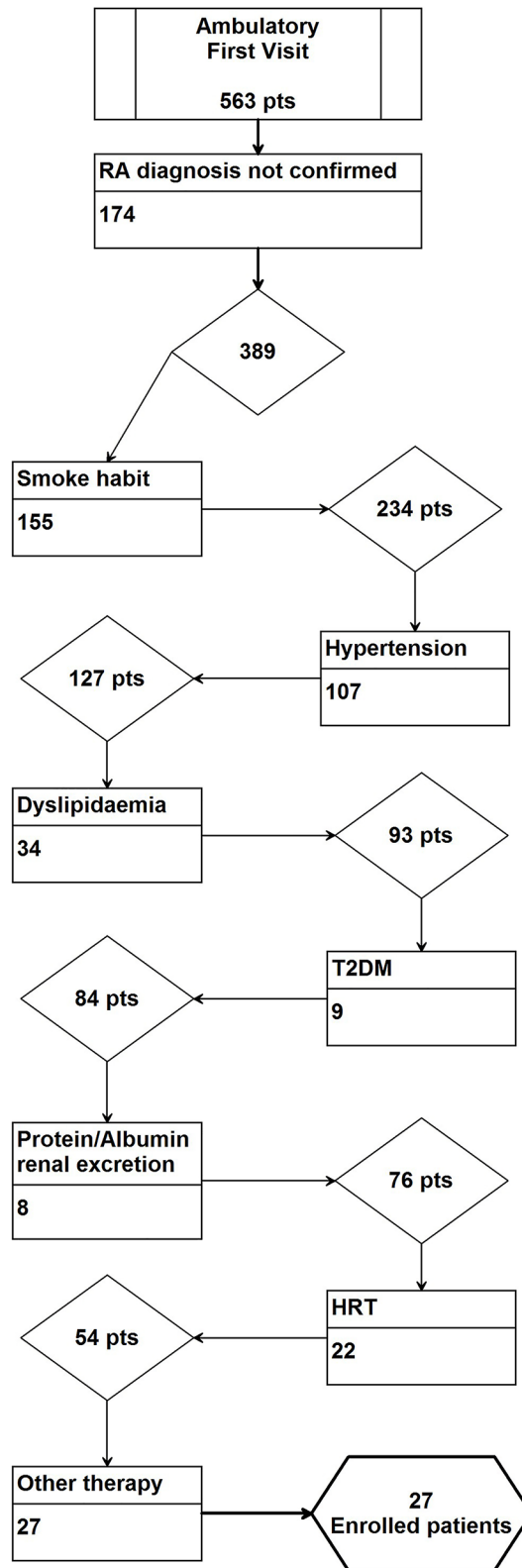


Fig 1. Flow diagram for patients exclusion.

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$DAS28 (CRP) = 0.56 * (TJC28)^{1/2} + 0.28 * (SJC28)^{1/2} + 0.014 * GH + 0.70 * \ln (CRP)$. Subjects with co-morbidities, such as diabetes mellitus (T2DM), dyslipidemia (defined as plasma levels of cholesterol ≥ 230 mg/dl or low-density lipoprotein cholesterol (LDL-C) ≥ 160 mg/dl, or triglycerides ≥ 250 mg/dl), hypertension (defined as systolic blood pressure (SBP) ≥ 140 mmHg and/or diastolic blood pressure (DBP) ≥ 90 mmHg) were excluded from the study. Smokers and patients previously supplemented with vitamin D analogues or derivatives were also excluded. Women taking hormone-based therapy were not included in the study. Thyroid, liver or kidney diseases, body mass index (BMI) ≥ 30 , alcohol consumption, abnormal electrocardiographic or echocardiographic (Left Ventricular Ejection Function, Left Ventricular Regional Function) pattern, and clinical history of CVD were also considered as exclusion criteria. To reduce the influence caused by seasonal variations in vitamin D status throughout the year, the participants were enrolled between October and May. Systematic pharmacological therapy was started after clinical and instrumental examinations.

Forty-one subjects (13 men and 28 women) matched for age and gender were enrolled from hospital personnel as control subjects. After inclusion in the study, patients and controls underwent blood sampling and instrumental examination as described below; patients were then referred to the rheumatology clinic for clinical and therapeutic follow-up.

Ethics Statement

Written informed consent was obtained from all subjects according to the Helsinki declaration and the observation was approved by the Ethics Committee of the University of Messina.

Methods

All chemical analyses were performed at the medical centre after an overnight fasting. Plasma lipids, glucose, fibrinogen, rheumatoid factor (RF) were determined by routine methods. CRP, anti-cyclic citrullinated peptide antibodies (aCCP) were determined by a commercially available ELISA kit.

25-hydroxyvitamin D3 (25-OH D) was measured using high-performance liquid chromatography (Bio-Rad Laboratories). According to Ross et al, Vitamin D deficiency was defined as 25-OH D levels < 20 ng/ml, insufficiency as 25-OH D levels between 20 and 30 ng/ml and optimal levels as 25-OH D levels > 30 ng/ml [32].

CD34+ cells identification and count

Flow cytometry (FACSCalibur; Becton Dickinson and Co., Franklin Lakes, NJ, USA) was used for cell identification. For this study, we identified and counted circulating CD34+ cells in peripheral blood. The cells were analyzed for the expression of surface antigens with direct multi-color analysis using fluorescein isothiocyanate (FITC)-conjugated, and phycoerythrin (PE)-conjugated monoclonal antibodies (mAbs) by flow cytometry analysis, as reported elsewhere [33]. Staining and analysis were performed using the International Society of Hematology and Graft Engineering (ISHAGE) sequential strategy [34]. All peripheral blood samples were collected and stored in 0.34 M K3EDTA anticoagulant and analyzed within 2 h. Fifty μ L of peripheral blood was then incubated with 10 μ L of FITC-conjugated anti-human CD45 mAb (Becton-Dickinson, BD, San Jose, CA, USA) and with 10 μ L PE-conjugated anti-human CD34 mAb (BD), using the multiparameter flow cytometric lyse no-wash method PROCOUNT (BD) in a TRUCOUNT tube (BD) with a known number of fluorescent beads (as reported on label of each BD TRUCOUNT lot); BD Pharm Lyse was used as Lysing Buffer. Incubation was performed at room temperature for 15 min in the dark. Next, 7-amino-actinomycin D (7-AAD; VIA-PROBE, BD Pharmingen, San Diego, CA, USA) was added to identify

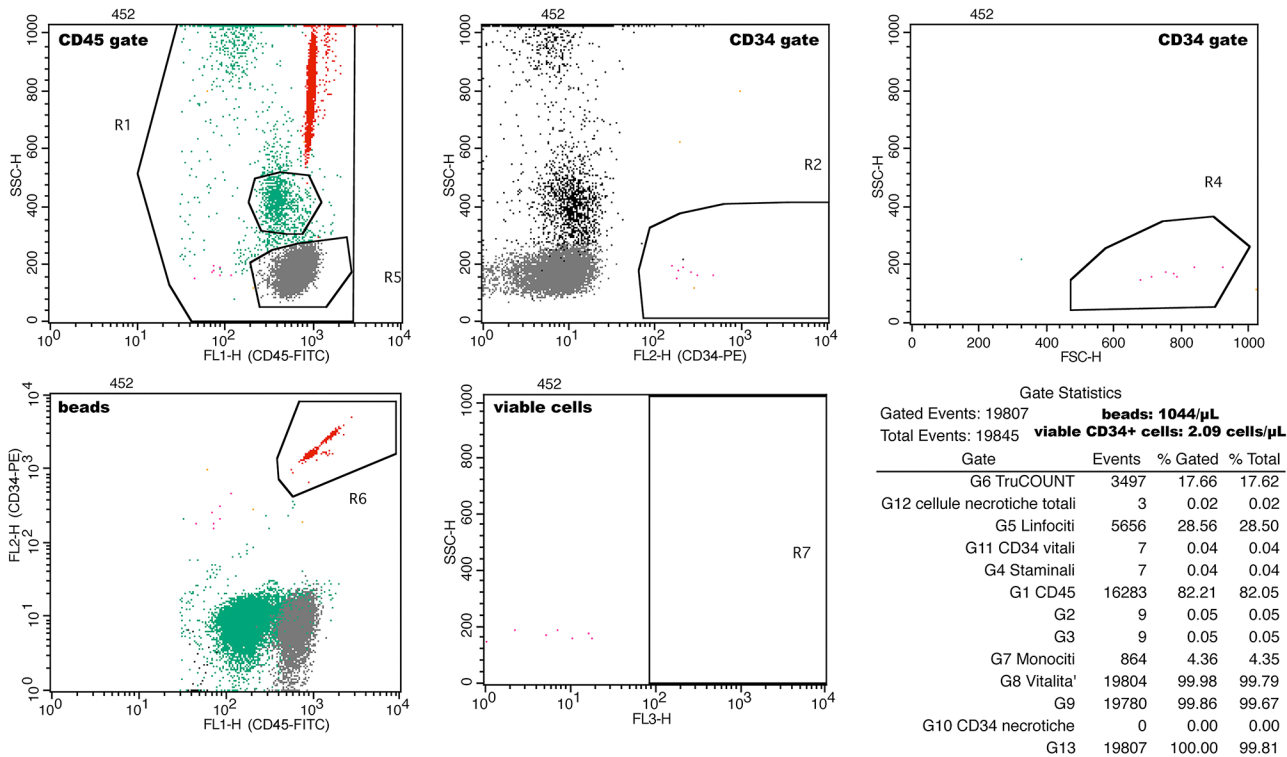


Fig 2. Flow cytometry sequential gating strategy.

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dead cells. To avoid cell loss, no wash was performed. Flow cytometric acquisition and analysis were performed by FACSCalibur; the threshold was set on FITC fluorescence in a dot plot of CD45-FITC vs side scatter (SSC) to exclude debris and ensure that all leukocyte populations and microbeads were included. Gating strategies and sample analyses allowed the identification of CD34+ cells by using the Macintosh CELLQuest software program (BD). Cell number was expressed as absolute count following manufacturer instructions, as reported below; during analysis, the absolute number (cells/μL) of positive cells in the sample can be determined by comparing cellular events to beads events, according to the formula: cell population absolute count = (number of event in cell populations/number of events in absolute count bead region)* (number of bead-test/test volume) (Fig 2). Only viable cells were counted; dead cells were excluded due to the use of 7-AAD. Consistently, viable CD34+ cells were identified and counted.

Measurement of cIMT and AS indices

Carotid echo Doppler scan and AS assessment were performed using a Vivid 3 Expert ultrasound machine equipped with a 7–15 MHz linear array transducer (GE Healthcare, Horten, Norway). cIMT has been expressed as the mean value of six-points IMT (common carotid, bifurcation, and internal carotid, bilaterally); according to ESC/ESH guidelines, we considered as carotid wall thickening a cIMT ≥0.9 mm [35], and carotid plaque ≥1.3 mm. PWV was determined by acquiring waveforms at the carotid and femoral arterial sites with electrocardiogram gating. Velocity (distance/time in m/s) was calculated by measuring the time interval between electrocardiogram R-wave and the recorded waveforms at each site, whereas distance between sites was measured manually.

The instrumental evaluations were repeated twice in duplicate by two experienced independent operators. The intraobserver/interobserver variability of IMT measurements was 0.01/0.02 mm; intraobserver/interobserver variability of PWV was 6.1/8.2%.

Statistical analysis

The Kolmogorov-Smirnov test verified that several variables had a non-normal distribution; consequently, we chose a traditional non-parametric approach (median and IQR) and, consistently, the variables were compared using the Mann-Whitney test. To increase the power of statistics, given also the small size of our sample, we chose to use the non-parametric combination test (NPCT), based on a permutation solution within a resampling procedure, as already suggested elsewhere [36]. Consistently, data are also shown as median \pm standard deviation (SD). The correlations among the variables were assessed by Spearman's test. To assess the contribution of each variable on study variables a linear, stepwise, multivariate regression analysis was performed that allows considering continuous and categorical variables together on the whole study population. A two-tailed alpha of 0.05 was used to denote statistical significance. SPSS statistical package (ver. 17.0, Chicago, IL), and NPC test 2.0 –Statistical software for multivariate permutation tests (Methodologica srl, Treviso, Italy) were used to perform statistical analyses.

Results

[Table 1](#) shows the baseline characteristics of the study groups. No difference was detected as regards age, BMI, gender, blood pressure, glucose, lipid profile. Fibrinogen, PWV and also cIMT values was significantly higher in RA patients compared to controls ($p < 0.001$) as was CRP ($p < 0.05$); moreover, cIMT was on average above 0.9 mm, considered the upper reference limit for preclinical atherosclerosis according to ESH-ESC guidelines [35]. Vitamin D levels and CD34+ cells were significant lower respect to controls (both $p < 0.001$). The disease activity score (DAS) and the duration of disease are also reported in [Table 1](#). Eighteen of 27 patients with RA were both RF and aCCP positive, nine were only RF positive. In RA, vitamin D insufficiency was found in 10 patients, vitamin D deficiency in 7 patients and sufficiency levels of vitamin D in 10 patients. [Fig 3](#) shows box and whiskers plots for CD34+ cell number (a) and vitamin D levels (b), in controls and RA patients. [Fig 4](#) shows box and whiskers plots for CD34+ cell number in RA patients according to have vitamin D deficiency (D), insufficiency (I) or optimal plasma levels (O).

We verified whether any correlation exists between CD34+ cell number, inflammatory markers (CRP, Fibrinogen), 25-OH D, and PWV in RA patients.

In RA patients we found a correlation between CD34+ cell number and vitamin D ($r_s = 0.706$, $p < 0.001$), fibrinogen ($r_s = -0.546$, $p < 0.001$), PWV ($r_s = 0.517$, $p < 0.01$) and cIMT ($r_s = 0.451$, $p < 0.05$), but not with CRP, DAS, or blood pressure values. A correlation was found between vitamin D and fibrinogen ($r_s -0.491$, $p < 0.01$), and with CD34+ cell number ($r_s 0.706$, $p < 0.001$), but no correlation was found between vitamin D serum levels and CRP, PWV, cIMT, lipids, glucose, blood pressure, RF, or aCCP.

A correlation was found in RA patients between CRP and PWV ($r_s = 0.633$, $p < 0.001$), but not with cIMT ($r_s = 0.125$, $p = 0.84$). Correlations among variables are summarized in [Table 2](#). [Fig 5](#) shows the correlations between CD34+ cell number and vitamin D levels (a) and fibrinogen (b), and between vitamin D levels and fibrinogen (c).

In order to assess the contribution of each variable on CD34+ cell number, we performed a multiple linear regression analysis; dependence analysis ([Table 3](#)) suggested that the main variable associated to CD34+ cell number was Vitamin D ($\beta = 0.567$; B: 0.060, 95%CI: 0.033; 0.087; $p < 0.001$), together with fibrinogen levels ($\beta = -0.323$; B: -0.060, 95%CI: -0.010; -0.002; p

Table 1. Characteristics of study population.

	RA		CONTROLS			
	Mean±SD	Median (IQR)	Mean±SD	Median (IQR)	NPCT	Mann-Whitney
Number	27		41			
Gender (m/f)	8/19		13/28			
Age (years)	47.5±12.5	47 (12)	46.4±4.1	46 (3)	0.474	0.577
BMI (kg/m ²)	25.2±4.2	25 (6.2)	23.8±2.5	23.5 (3.6)	0.12	0.08
SBP (mmHg)	125.2±10.3	125 (15)	121.6±9.1	120 (18)	0.13	0.16
DBP (mmHg)	73.1±11.9	70 (15)	70.7±6.3	70 (10)	0.15	0.12
TC (mg/dl)	191.9±43	192 (75)	181.9±21	180 (36.5)	0.08	0.21
HDL-C (mg/dl)	63.5±14.9	61 (20)	54.5±10.3	53 (9)	0.002	0.004
TG (mg/dl)	109.9±60.8	81 (67)	108±20.8	103 (22.5)	0.8	0.11
LDL-C (mg/dl)	109.5±40.8	100 (61.6)	108.1±32.5	108.2 (37.8)	0.8	0.8
Glucose (mg/l)	94.5±17.3	94 (16)	88.2±6.7	88 (11)	0.08	0.12
Hs-CRP (mg/dl)	0.9±1.1	0.65 (1.1)	0.4±0.22	0.4 (0.3)	<0.001	<0.05
Fibrinogen (mg/dl)	336.6±61	325 (81)	271.9±56	291 (95.5)	<0.001	<0.001
DAS 28	4.1±0.75	3.8 (1.37)	-	-	-	-
Duration disease (months)	5.1±3.9	4 (3)	-	-	-	-
25-OHD (ng/ml)	23±7.6	24 (15.1)	31.7±5.2	32 (7.20)	<0.001	<0.001
CD34+ (cells/μL)	1.8±0.6	1.7 (1.20)	2.5±0.9	2.46 (1.85)	0.0010	<0.01
PWV (m/s)	8.38±2.43	7.55 (4.22)	4.73±0.74	4.95 (0.75)	<0.001	<0.001
cIMT (mm)	1±0.17	1.1 (0.25)	0.76±0.13	0.71 (0.2)	<0.001	<0.001

Values are median (IQR) or mean ± SD. Abbreviations: BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; HDL-C: high density lipoprotein-cholesterol; TG: triglycerides; LDL-C: low density lipoprotein cholesterol; Hs-CRP: high sensitivity C-reactive protein.; DAS 28: Disease Activity Score; PWV: pulse wave velocity; cIMT: carotid intima-media thickness. p: p value level for two tailed Mann-Whitney test or NPCT, RA patients versus controls.

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<0.005); the model estimated for vitamin D assessed as statistical predictors both CD34+ cell count ($\beta = 0.389$; B: 3.100, 95%CI: 1.535; 4.665; $p < 0.001$), and fibrinogen ($\beta = -0.368$; B: -0.042, 95%CI: -0.064; -0.020; $p < 0.001$).

Discussion

Considerable evidence indicates that patients with RA have an increased risk of developing CVD [1, 3]. The proinflammatory status occurring in RA is characterized by activation of inflammatory cells, increased levels of inflammatory mediators and the presence of autoantibodies, concurring in inducing endothelial dysfunction, vascular wall stiffening and thickening, and premature atherosclerosis [2, 4]. Endothelial damage can be counteracted by endothelial repair, and circulating progenitor cells originating from the bone marrow may contribute at least in part to this process [5, 10, 37]. Several cell phenotypes have been proposed as EPCs and considered as potential spare cells, able to participate in the turnover of healthy and damaged vascular endothelium; low EPCs number has been shown to correlate with a higher incidence of CV events [9, 38], supporting the notion that EPCs are relevant in the pathophysiology of CVD. Although the question of which cell phenotype better identifies the “true” circulating EPC is to date unsolved [10, 11], by evaluating the impact of different CPC immunophenotypes on the ability to predict or associate with CV risk and outcomes, it has been suggested that CD34+ cell count may be more closely linked to CV risk than CD133+ cell number and multiple positive phenotypes, underlining that a more extensively characterization of

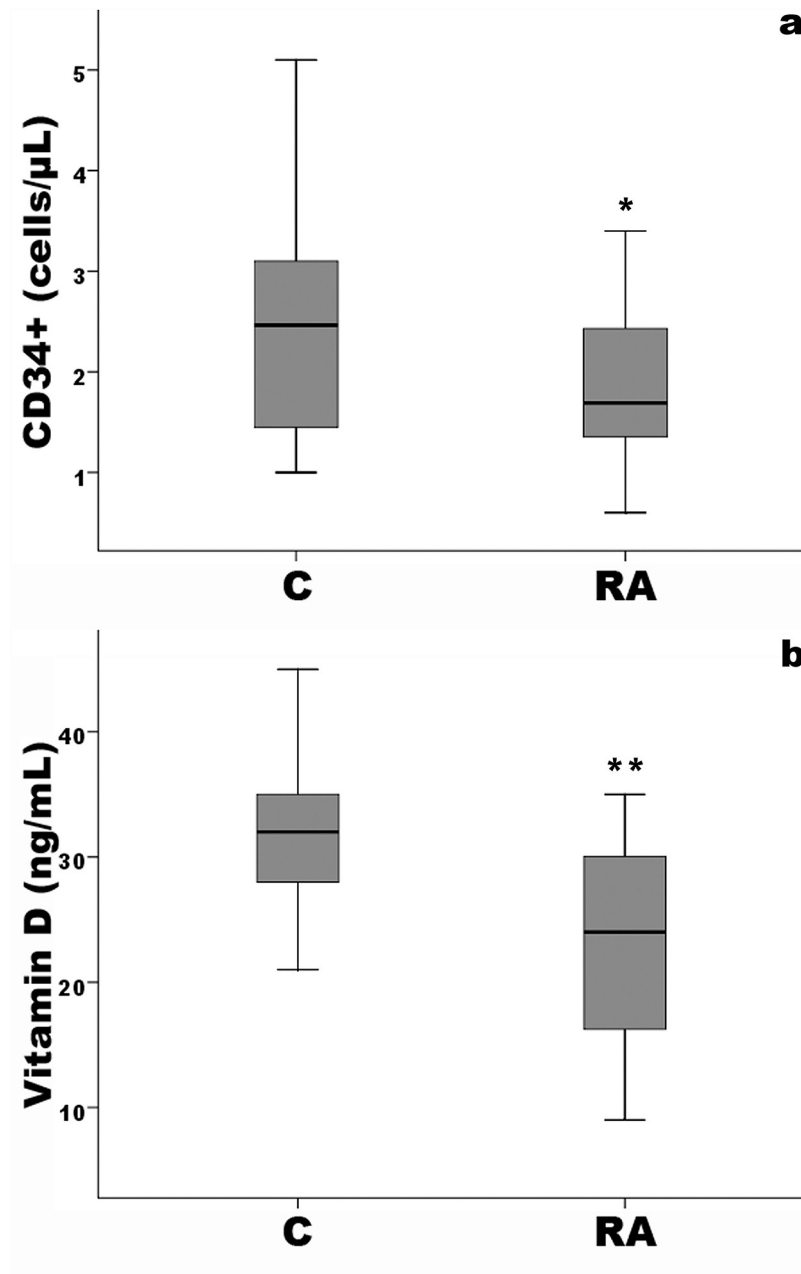


Fig 3. Box and whiskers plots for CD34+ cell number (a) and Vitamin D levels (b) in controls (C) and RA patients (RA). * $p < 0.01$ vs controls, ** $p < 0.001$ vs controls. Solid horizontal lines = median values; error bars = 95% Confidence intervals; Shaded area = Interquartile range.

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circulating CD34+ cells may not be useful for this purpose [14, 15]. The surface antigen CD34, in fact, appears to identify a cluster of cells with progenitor and stem activity, and, in many cases, CD34+ population shows an important differentiation and also transdifferentiation ability [12, 13]. Consistently, CD34+ cell count has been often proposed as prognostic marker in several different clinical settings [5, 6, 9–12, 14, 15, 21].

Indeed, it has been suggested that in RA patients the impairment of endothelial repair mechanisms may be at least in part due to the altered number or function of PHCs; in addition,

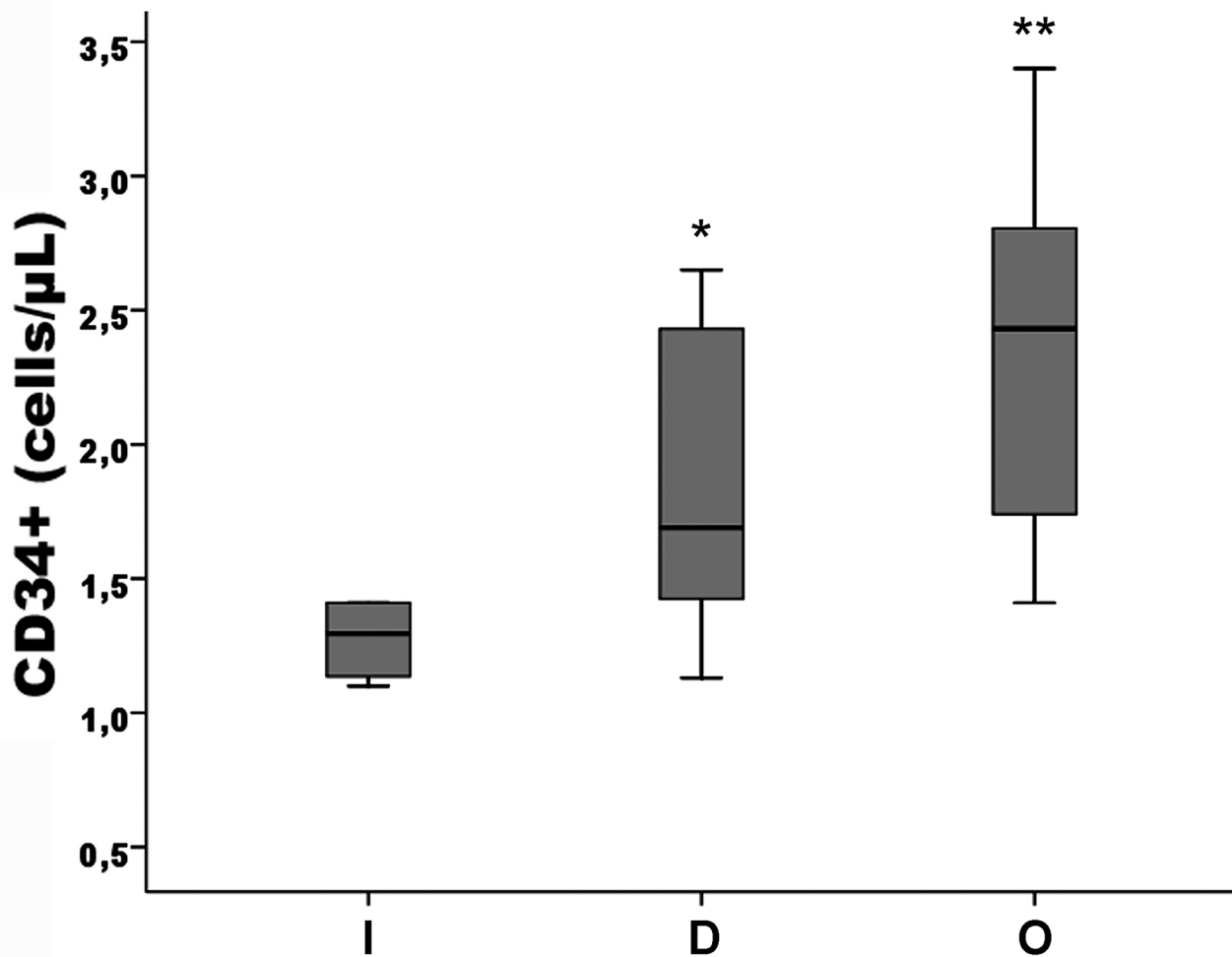


Fig 4. Box and whiskers plots for CD34+ cell number in RA patients with insufficient (I), deficient (D), and optimal (O) vitamin D levels. *p<0.01 vs controls, **p<0.001 vs controls. Solid horizontal lines = median values; error bars = 95% Confidence intervals; Shaded area = Interquartile range.

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Table 2. Relationships among the variables.

Whole population	BMI	TC	HDL-C	CRP	Fibrinogen	PWV	cIMT	25-OH D
CD34+ cell number	rs -0.251	rs -0.247	rs -0.112	rs -0.147	rs -0.197	rs -0.302	rs -0.290	rs 0.493
	p = 0.031	p = 0.034	p = ns	p = ns	p = ns	p = 0.017	p = ns	p <0.001
25-OH D	rs -0.234	rs -0.126	rs -0.259	rs -0.252	rs -0.371	rs -0.551	rs -0.408	
	p = 0.045	p = ns	p = 0.026	p = 0.030	p <0.001	p <0.001	p = 0.043	
RA only	BMI	TC	HDL-C	CRP	Fibrinogen	PWV	cIMT	25-OH D
CD34+ cell number	rs -0.151	rs -0.139	rs -0.252	rs -0.092	rs -0.546	rs 0.517	rs 0.451	rs 0.706
	p = ns	p = ns	p = ns	p = ns	P<0.001	p = 0.006	p = 0.018	p <0.001
25-OH D	rs -0.166	rs -0.134	rs -0.187	rs -0.203	rs -0.491	rs 0.207	rs 0.326	
	p = ns	p = ns	p = ns	p = ns	p = 0.009	p = ns	p = ns	

Correlations among the variables; upper panel: whole population; lower panel: RA patients only. rs: Spearman's coefficient; p: level of significance of rs.

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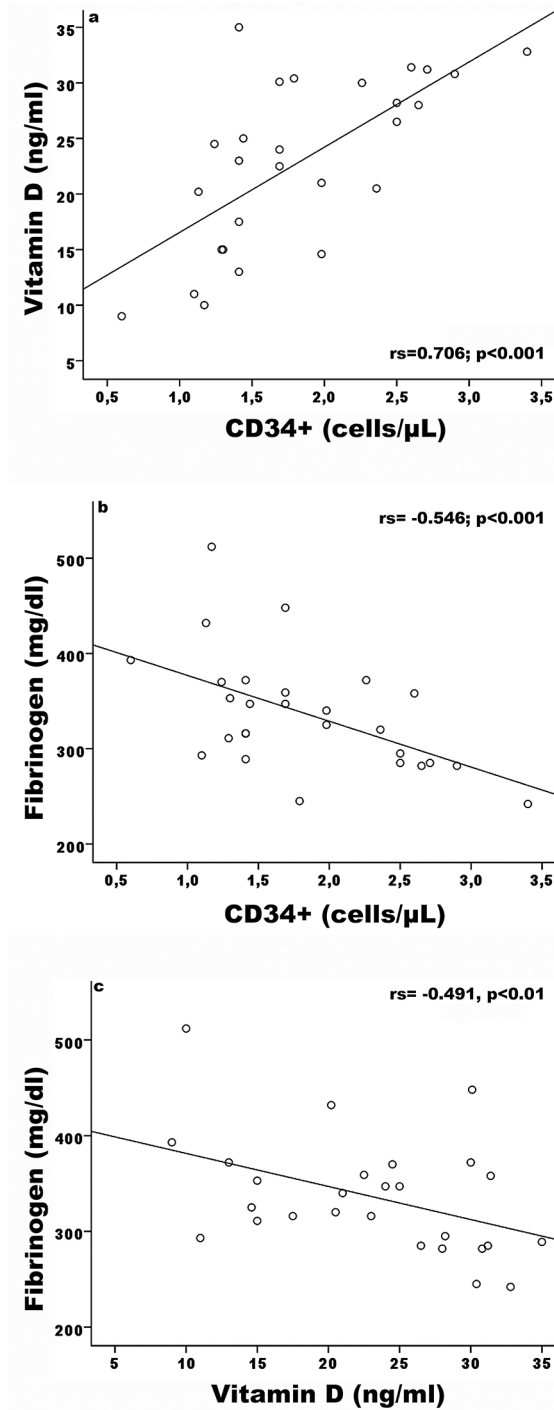


Fig 5. Spearman's correlation between: CD34+ cell number and Vitamin D levels (a), Fibrinogen (b); Vitamin D levels and Fibrinogen (c). RA subjects. Spearman's coefficient (r_s) is shown with its significance (p).

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Table 3. Multiple regression analysis.

Dependent variable	Predictors	B	95% CI for B	Beta	T	P
CD34+ cell number	25-OH D	0.060	0.033; 0.087	0.567	4.573	<0.001
	Fibrinogen	-0.060	-0.010; -0.002	-0.323	-3.624	<0.005
25-OH D levels	CD34+ cells	3.100	1.535; 4.665	0.389	3.949	<0.001
	Fibrinogen	-0.042	-0.064; -0.020	-0.368	-3.736	<0.001

Multiple regression analysis for CD34+ cell number, and 25-OH D levels, in RA subjects. B, unstandardized regression coefficient; 95%CI, 95% Confidence Interval for B; Beta, standardized regression coefficient; T, t-test for Beta; p, p-value for significance.

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a novel T cell subset, the so-called angiogenic T cells (Tang), has been more recently suggested to cooperate with circulating progenitors. To date, the relationships between Tang and the different subsets of circulating progenitors are not at all defined, as were their prognostic value in the different clinical settings, but it has been suggested that Tang may be needed to enhance angiogenic properties and endothelial repair function of CD34+ cells, possibly through the secretion of proangiogenic cytokines [16–18].

According with previous studies [21, 22], we confirm a reduction of CD34+ cells in RA patients which might be associated to the chronic inflammatory status. It is known, in fact, that a loss of function and apoptosis of CD34+ cells occur during chronic inflammatory diseases [39]; more recently, it has been also shown a direct link between high α -interferon, low EPC count and increased CV risk [40]. Also, anti-TNF α therapy seems able to restore CPC number in juvenile arthritis [41].

In addition, as already recently reported, we found low levels of Vitamin D in RA patients [27, 42]. In RA patients with moderate disease activity we found an interdependence between CD34+ cell number and early atherosclerosis, as defined by PWV increase and carotid intima-media thickening; CD34+ cell number, also, appears to be affected by low 25-OH D levels.

Vitamin D deficiency is a condition independently associated to adverse CV events [24], and lower serum 25-OH D is associated to inflammation-linked vascular endothelial dysfunction [25] and coronary artery disease, but not to peripheral arterial disease or arterial stiffening [26]. Vitamin D might have a role in the proliferation and differentiation of stem cells, also toward endothelial phenotype, by modulating phospholipase C, VEGF1 and 2, and pro-MMP-2 activity [30, 43]. In addition, it was also proposed that vitamin D might protect PHCs from oxidative stress [44]; in fact, as we previously showed, CD34+ cells from RA patients have increased intracellular ROS levels and imbalanced antioxidant enzymes [22] that could explain at least in part the reduced PHC number found in these patients. Consistently, RA patients with higher vitamin D levels presented with higher CD34+ cell number.

Vitamin D deficiency is commonly associated with inflammatory diseases; this association could be due to an increased prevalence of inflammatory diseases in hypovitaminosis D, although reverse causality cannot be excluded [45]. We also observed a correlation between vitamin D levels and inflammatory markers such as fibrinogen. Reid et al reported that plasma concentrations of 25-OH D decrease after an inflammatory insult, and this decrease appears to persist for several months [46]. However, the relationships with inflammatory markers and the potential role of vitamin D appear to be controversial; in a recent analysis Amer and Qayyum confirmed an inverse relation between vitamin D and CRP at lower levels only, while higher serum levels of vitamin D appear to be associated with higher levels of CRP at least in asymptomatic subjects [47]. In RA it has already been described an association between vitamin D levels and erythrocyte sedimentation rate (ESR), disease activity scores, and also with IL-17 and IL-23 serum levels [48]. Also, Ranganathan et al confirmed an association between vitamin

D insufficiency and serum levels of IL-17 in patients with RA [42]. Furthermore, Vitamin D and its analogues inhibit IL-2 and IFN- γ production and stimulates the effects of T-helper type 2 lymphocytes, leading to a reduction in MMPs, and delaying atherosclerotic plaque progression [49]. Vitamin D supplementation has been already proposed as adjuvant treatment for neurological, cardiovascular, respiratory and metabolic diseases, as well in chronic inflammatory diseases; however, the role of vitamin D supplementation in modifying the clinical outcomes has not been definitely confirmed [50–54], and it remains unclear whether vitamin D status is causally related to the pathogenesis of the diseases or is merely a marker of health [55].

We also confirmed an increased carotid AS in RA patients with moderate disease activity, which do not appear to be directly associated to serum vitamin D levels. The role of vitamin D in vascular stiffening is supposed to be multifactorial, and different plausible mechanisms may explain how vitamin D is involved in cardiometabolic outcomes; there is evidence about the implication of vitamin D in regulating renin angiotensin system, proliferation of vascular smooth muscle, insulin resistance, anticoagulant activity, macrophage activation and cytokine generation [23, 24].

Our results, although do not provide a defined mechanism by which vitamin D may interact with CD34+ cells and fibrinogen, suggest that vitamin D could be involved in endothelium homeostasis.

This study however presents several limitations. The first limitation is the small sample size; according to inclusion/exclusion criteria, in fact, we enrolled 27 patients only with moderate disease activity. Second, we investigated the association between inflammation, vitamin D levels and CD34+ cells without a focused investigation on mechanistic and pathophysiological aspects that could explain this association, but this was not the purpose of the study.

In conclusion, our findings confirm Vitamin D deficiency in RA patients, and its association with inflammation; moreover, we suggest that CD34+ cell number is reduced in RA patients with moderate disease activity, mainly in subjects with lower vitamin D levels.

It is likely that this novel association may, at least in part, contribute to explaining the increase of CV morbidity and mortality in patients suffering from RA. Further studies on larger sample sizes could clarify whether a supplementation of Vitamin D could modify CD34+ levels and inflammatory indices in patients affected by rheumatoid arthritis and therefore contributing to reduce cardiovascular risk in this patients.

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Author Contributions

Conceived and designed the experiments: ALG GM GB COA AS. Performed the experiments: ALG RLG COA EI ADA AGV SL AC. Analyzed the data: GM EM. Contributed reagents/materials/analysis tools: ADA FR SL CS. Wrote the paper: ALG GM MAS.

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