ORIGINAL RESEARCH

Cumulative Rheumatic Inflammation Modulates the Bone–Vascular Axis and Risk of Coronary Calcification

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***Background*—**Rheumatic diseases are related to both abnormal bone turnover and atherogenesis, but a mechanistic link was missing.

***Methods and Results*—**We investigated the effect of cumulative rheumatic inflammation (CRI) on risk of coronary calcification in a retrospective cohort of 145 rheumatoid arthritis patients. A time-adjusted aggregate CRI score was derived by conglomerating all quarterly biomarker encounters of serum C-reactive protein over 60 months immediately preceding computed tomography coronary angiography. Flow cytometry was performed to measure the osteocalcin-positive (OCN^+) $CD34^+KDR^+$ and OCN^+CD34^+ circulating endothelial progenitor cells (EPCs). Conventional early circulating EPCs $CD34^+CD133^+KDR^+$ was determined. Coronary calcification was defined as any Agatston score >0. 50% of patients (n=72/145) had coronary calcification. CRI score was associated with presence of coronary calcification (P=0.004) (multivariable-adjusted: highest versus lowest quartile: odds ratio=5.6 [95% CI 1.1–28.9], P=0.041). Receiver operating characteristics curve revealed divergent behavior of OCN-expressing circulating EPCs (OCN^+CD34^+ EPCs: area under the curve=0.60, P=0.034; $OCN^+CD34^+KDR^+$ EPCs: area under the curve=0.59, P=0.053, positive predictors) versus conventional early EPCs ($CD34^+CD133^+KDR^+$: area under the curve=0.60, P=0.034, negative predictor) for coronary calcification, which persisted after multivariable adjustments ($OCN^+CD34^+KDR^+$ [>75th percentile]: odds ratio=7.2 [95% CI 1.8–27.9], P=0.005; OCN^+CD34^+ EPCs [>75th percentile]: odds ratio=6.0 [95% CI 1.5–23.3], P=0.010; $CD34^+CD133^+KDR^+$ [>75th percentile: odds ratio=0.3 [95% CI 0.1–1.0], P=0.053). Intriguingly, the CRI score was associated with increased OCN^+CD34^+ EPCs (highest versus lowest quartile: B=+25.6 [95% CI 0.8–50.5] [$\times 10^3$/mL peripheral blood], P=0.043), but reduced $CD34^+CD133^+KDR^+$ EPCs (highest versus lowest quartile: B=−16.2 [95% CI −31.5 to −0.9], P=0.038).

***Conclusions*—**Preceding 60 months of CRI is associated with increased risk of coronary calcification and altered OCN expression in circulating EPCs. **(*J Am Heart Assoc*. 2019;8:e011540. DOI: 10.1161/JAHA.118.011540.)**

Key Words: coronary artery disease • endothelial progenitor cells • inflammation • osteogenesis • rheumatoid arthritis

Circulating endothelial progenitor cells (EPCs) play a pivotal role in mediating vascular repair, such that in response to acute vascular insults, they mobilize from the bone marrow and host to sites of vascular injury and mediate a reparative process through enhanced re-endothelialization.[1] However, it became increasingly apparent that the bone marrow did not merely serve as a submissive cradle for these progenitor cells, but there were pathophysiological crosstalks between abnormal bone metabolism and atherogenesis.[2,3]

Studies revealed that there is a heterogeneous overlap of phenotypes between endothelial versus osteoblastic progenitor cell lineages, such that a disrupted balance of their interplay may result in abnormal vascular repair and accelerated calcification,[4] exacerbated under various systemic driving factors including aging and hormonal changes, hyperglycemia, effect of medications, as well as systemic inflammatory stress.[5–9] The endothelial-to-mesenchymal transition provocative of atherosclerosis and plaque instability could be activated through the activin-like kinase-2 receptor-dependent mechanism,[10,11] which is also linked to inflammation.[12] The role of systemic inflammation in modulating this bone–vascular axis remained unclear and may be of particular

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Clinical Perspective

What Is New?

- This study revealed that preceding 60 months of cumulative rheumatic inflammation is associated with increased risk of coronary calcification and altered osteocalcin expression in circulating endothelial progenitor cells.

What Are the Clinical Implications?

- Inflammation-driven modulation of the bone–vascular axis may play an important pathophysiological role in coronary calcification among rheumatic patients, warranting further definitive experimental mechanistic studies.

relevance in inflammation-prone subjects. While patients with rheumatism such as rheumatoid arthritis (RA) are at notoriously increased risks of both atherosclerosis and abnormal bone turnover (namely, bone erosion and osteoporosis),[13] how the osteoblastic activity and in turn coronary calcification risk are influenced by rheumatic inflammatory load had never been studied.

We therefore sought to investigate how cumulative rheumatic inflammation impacts on the bone–vascular axis and risk of coronary calcification. As part of the atherosclerotic cardiovascular continuum, arterial stiffness is assessed conjunctively.

Methods

Study Subjects

We conducted a clinical–pathophysiological retrospective cohort study of inflammation-predisposed patients diagnosed with rheumatoid arthritis (RA) (n=145), based on the American College of Rheumatology (prior American Rheumatism Association) classification criteria.[14] All patients were recruited consecutively from internal medicine clinics during the period from March 2015 to November 2016. Comprehensive demographic and clinical data were documented and ascertained. Inflammatory biomarkers and relevant clinical data 60 months preceding the indexed recruitment date were retrieved from records of the central Clinical Management System, the territory-wide computerized clinical data network of all public hospitals in Hong Kong. Among them, 61 (42%) had hypertension, 9 (6.2%) had diabetes mellitus, and 68 (47%) had hyperlipidemia. Only 5 patients (3.4%) had a pre-existing history of coronary artery disease and they were intentionally retained in the random sample to avoid exclusion bias. Fasting blood was collected for biochemical analysis of serum low-density lipoprotein-cholesterol, triglycerides, high-density lipoprotein-cholesterol, glycated hemoglobin, creatinine, erythrocyte sedimentation rate, and CRP (C-reactive protein). Patients with the following conditions were excluded: recent myocardial infarction, unstable angina, coronary revascularization, and stroke or acute heart failure within the past 6 months, dilated cardiomyopathy, significant valvular heart disease, New York Heart Association class III or IV heart failure, and liver failure or significant renal impairment with creatinine >220 mmol/L. All patients gave written informed consent. The study was approved by the institutional Ethics Committee and carried out in accordance with the Declaration of Helsinki. Data sets analyzed and/or generated during this study are not made publicly available, because the act of data sharing had not been included as part of the original research protocol at the time of Ethics Committee approval. The authors therefore made the best effort in presenting the comprehensive and relevant data in this article.

Flow Cytometry

Fluorescence-activated cell analysis was performed to determine osteocalcin (OCN) expression and numbers of peripherally circulating EPCs.

The number of OCN-positive (OCN^+) $CD34^+KDR^+$ and OCN^+CD34^+ circulating EPCs was measured and expressed in absolute numbers (unit in $\times 10^3$/mL peripheral blood) and percentages of total peripheral blood mononuclear cells. Conventional early circulating EPCs $CD34^+CD133^+KDR^+$ was determined. $CD34^+CD133^+KDR^+$ cells may represent less differentiated EPCs,[15] and have been associated with bone turnover markers in previous studies.[6] Increased circulating EPCs was defined as any level beyond 75th percentile of the study population. Briefly, blood samples were aliquoted into 4 portions for preservation under −70°C before flow cytometry. Each 100 μL of peripheral blood was incubated with a fluorescein isothiocyanate–conjugated antibody targeting at CD34 and CD133, respectively (Beckman Coulter, Fullerton, CA). A human OCN phycoerythrin-conjugated antibody (R&D Systems) was used to identify OCN^+ cells. Fluorescein isothiocyanate–labeled anti-human CD45 antibody was used for differential gating during flow analysis. Fluorescein isothiocyanate–labeled IgG1a (Beckman Coulter) and phycoerythrin-labeled IgG2b (Becton Dickinson, Franklin Lakes, NJ) served as isotypic controls for color compensation. Analysis was performed with an automated fluorescence-activated cell counter (Elite, Beckman Coulter), during which 1 000 000 events were counted. The absolute cell counts of all the measured components per 1 000 000 events in the lymphocyte gate were calculated.

Computed Tomography Assessment of Coronary Calcification

Computed tomography (CT) assessment of the coronary arteries was performed for all subjects using a 64-slice

multidetector CT (Lightspeed, VCT, GE Healthcare), as previously described.[16,17] Imaging was performed with subjects resting in the supine posture, and included regions from the aortic arch to the fundus of the heart with prospective ECG gating (rotation time=0.35 seconds, slice thickness=2.5 mm; 120 kV; 250 mA; trigger delay=70% R-R interval). Patients were instructed to hold their breath for 30 seconds during image acquisition. Acquired images were analyzed offline at the postprocessing workstation (Advantage windows 4.02, GE Healthcare). Images with motion artifacts or asynchronous electrocardiographic triggering were excluded. Two independent investigators blinded to subject clinical status separately analyzed all scans. The interobserver and intraobserver variability correlation coefficients of coronary calcification measurements from our group were 0.92 and 0.91, respectively. The coronary calcification score was assessed using the software "smart score" (GE Healthcare) with threshold set for pixels >130 Hounsfield units and expressed in Agatston units, and was derived from the sum of calcium scores in the left main coronary artery, left anterior descending artery, left circumflex coronary artery, right coronary artery, and posterior descending artery. Presence of coronary calcification was defined as any coronary calcium score >0.

Cumulative Inflammation Load

Data on serum CRP was retrieved for each subject from each quarterly measurement in the past 60 months immediately preceding the date of CT coronary angiography and EPCs measurements, thus yielding a total of 20 consecutive biomarker encounters for each subject. Raised CRP was defined as >0.35 mg/dL. A constituent score of 1 was assigned to each biomarker encounter with any raised CRP level >0.35 mg/dL, whereas any biomarker encounter with a CRP level ≤0.35 mg/dL was conferred a constituent score of 0. A Cumulative Rheumatic Inflammation (CRI) score (0–20) was thus generated. Up to 88.3% of subjects had at least 60% of complete data on 20 biomarker encounters. All CRI score estimates were time-adjusted to duration of biomarker data availability to ensure internal validity for comparison.

Assessment of Arterial Stiffness

As previously described,[18,19] arterial stiffness was noninvasively measured using VP-2000 System (Colin Corp., USA). Supine resting for 5 minutes was ensured before measurement. Sequential pressure waveforms at the precordium and posterior tibial arteries were respectively studied using handheld manometer probes and blood pressure cuffs with synchronized ECG gating. Assessment took place after coherently reproduced signals with maximum amplitudes. Brachial–ankle pulse wave velocity (baPWV) was calculated as the distance between measurement points over the respective brachial and tibial arteries, divided by pulse transit time between the systolic R-wave and upstroke of waveform at the respective tibial artery. Calculation was performed using system software with R-wave synchronization beyond 10 cardiac cycles with averaging of left- and right-sided derived estimates. Intraobserver variability testing from our group revealed an intraclass correlation coefficient of 0.87 ($P<0.001$) (2-way mixed, random-effect model, absolute agreement).

Sample Size Calculations

Because osteogenic circulating EPCs were the primary parameter of interest, based on the magnitude of differences in OCN^+ EPCs detected in patients with stable coronary artery disease versus those with at least 1 cardiovascular risk factor,[3] a total study sample comprising 128 subjects (n=64 subjects respectively with, or without coronary disease; sampling ratio 1:1) has 80% power to reject the null hypothesis at a Type I error of 0.05. Based on an additional study[20] that showed that patients with psoriasis had reduced circulating conventional early EPCs ($CD133^+/KDR^+$), which was another key parameter of interest, assuming Type I error=0.05, a minimal sample size of 64 subjects provides a study power of 99% to detect significant between-groups differences in circulating conventional EPCs. Our sample size of 145 was determined pragmatically in this retrospective study, based on patient data availability in our specialty clinic center.

Statistical Analysis

Relations between variables of interest and coronary calcifications were examined by Student t test and χ^2 test, as appropriate. Strengths of linear associations were expressed by Pearson correlation coefficient. Receiver-operating characteristics curves were used to analyze the performance of OCN-expressing $OCN^+CD34^+KDR^+$ and OCN^+CD34^+ EPCs and conventional circulating $CD34^+CD133^+KDR^+$ EPCs, as well as CRI inflammatory score for predicting development of coronary calcification. Area-under-the-curve (AUC) was presented as a unified estimate of sensitivity and specificity. Univariable logistic regression was used to examine the unadjusted associations between variable of interest and coronary calcification. A fully adjusted multivariable logistic regression model was used to estimate the adjusted odds ratio (OR) for each of the EPCs cell lineages and cumulative inflammation load for coronary calcification, in which each potentially confounding variable a priori defined based on demographic or pathophysiological grounds was entered: age, sex, history of

smoking, systolic and diastolic blood pressure, resting heart rate, fasting levels of low-density lipoprotein-/high-density lipoprotein-cholesterol, triglycerides, glycated hemoglobin, arterial stiffness, creatinine, use of statins and disease-modifying antirheumatic agents, and duration of RA. Univariable and multivariable linear regression models were

Table 1. Clinical Characteristics of Subjects Stratified by Presence of Coronary Calcification

	Normal Coronary (n=73)	Coronary Calcification (n=72)	*P* Value
Male, n (%)	4 (5.5)	14 (19.4)	0.003*
Age, y	57.4±9.4	64.6±11.1	<0.001*
Smoking, n (%)	6 (8.2)	9 (12.5)	0.40
Diabetes mellitus, n (%)	3 (4.1)	6 (8.3)	0.29
Hypertension, n (%)	22 (30.1)	39 (54.2)	0.003*
Hyperlipidemia, n (%)	32 (44.4)	36 (50.0)	0.50
Systolic blood pressure, mm Hg	123.4±19.8	133.3±19.7	0.003*
Diastolic blood pressure, mm Hg	74.5±12.7	77.6±11.5	0.13
Resting heart rate, beats/ min	66.5±11.5	66.7±9.4	0.90
LDL-cholesterol, mmol/L	2.6±0.7	2.7±0.8	0.86
HDL-cholesterol, mmol/L	1.7±0.4	1.6±0.5	0.15
Triglycerides, mmol/L	1.1±0.6	1.3±0.7	0.15
HbA1c, %	5.3±0.4	5.7±0.8	<0.001*
Creatinine, μmol/L	63.4±13.4	71.0±16.7	0.003*
Statin use, n (%)	14 (19.4)	23 (31.9)	0.086
DMARDs use, n (%)			
Hydroxychloroquine	45 (61.6)	45 (62.5)	0.92
Methotrexate	54 (74.0)	48 (66.7)	0.34
Sulfasalazine	36 (49.3)	22 (30.6)	0.021*
Leflunomide	15 (20.5)	13 (18.1)	0.70
RA disease duration, mo	12.8±6.1	14.2±7.2	0.20
CRP, mg/dL	0.56±0.51	0.80±0.76	0.027*
ESR, mL/h	36.7±19.0	44.8±26.5	0.035*
Aggregate CRI score	0.10±0.10	0.16±0.12	0.004*
Brachial–ankle PWV, cm/s	1475.1±321.6	1701.3±364.7	<0.001*
Circulating osteocalcin-positive EPCs			
$OCN^+CD34^+KDR^+$			
Absolute number, $\times 10^3$/mL	19.77±20.48	24.92±18.90	0.12
% in PBMC	0.16±0.12	0.21±0.14	0.041*
OCN^+CD34^+			
Absolute number, $\times 10^3$/mL	33.73±34.82	48.05±44.52	0.034*
% in PBMC	0.27±0.24	0.41±0.42	0.020*
Circulating conventional early EPCs			
$CD34^+CD133^+KDR^+$			
Absolute number, $\times 10^3$/mL	29.63±29.19	20.45±19.81	0.029*
% in PBMC	0.26±0.27	0.18±0.19	0.031*

CRI indicates cumulative rheumatic inflammation; CRP, C-reactive protein; DMARDs, disease-modifying antirheumatic agents; EPCs, endothelial progenitor cells; ESR, erythrocyte sedimentation rate; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OCN, osteocalcin; PBMC, peripheral blood mononuclear cell; PWV, pulse-wave velocity; RA, rheumatoid arthritis.
*$P<0.05$.

used to derive the crude and adjusted prediction estimate (expressed as B with 95% CI) of CRI score on cell lineages of OCN-expressing and conventional circulating EPCs. All analyses were performed using SPSS Statistics (Version 21). $P<0.05$ was considered statistically significant.

Results

Clinical characteristics of subjects are presented in Table 1. Fifty percent of subjects (n=72/145) had CT-detected coronary calcification. Coronary calcification was associated with higher age ($P<0.001$), male sex ($P=0.003$), history of hypertension/systolic blood pressure ($P=0.003$), higher glycated hemoglobin ($P<0.001$), and serum creatinine ($P=0.003$). Furthermore, coronary calcification was positively associated with baPWV (Pearson $R=0.28$, $P=0.001$).

EPCs OCN Expression and Coronary Calcification

As shown in Table 1, the percentage of OCN-expressing circulating $OCN^+CD34^+KDR^+$ ($P=0.041$) and OCN^+CD34^+ EPCs ($P=0.020$) in peripheral blood was significantly higher in patients with CT-detected coronary calcification. Conversely, these patients with coronary calcification had significantly lower circulating conventional early EPCs $CD34^+CD133^+KDR^+$ (absolute number, $P=0.029$; % of peripheral blood mononuclear cells, $P=0.031$). Strikingly, receiver-operating characteristics curve analyses revealed divergent behavior of OCN-expressing circulating EPCs (OCN^+CD34^+ EPCs: AUC=0.60, $P=0.034$; $OCN^+CD34^+KDR^+$ EPCs: AUC=0.59, $P=0.053$, positive predictor, Figure 1A) versus conventional early EPCs ($CD34^+CD133^+KDR^+$: AUC=0.60, $P=0.034$, negative predictor, Figure 1B) in the risk prediction for coronary calcification development. After multivariable adjustment for potential confounders (Table 2), OCN-expressing EPCs remained independently associated with increased risk of coronary calcification ($OCN^+CD34^+KDR^+$ EPCs [>75th percentile]: OR=7.2 [95% CI 1.8–27.9], $P=0.005$; OCN^+CD34^+ EPCs [>75th percentile]: OR=6.0 [95% CI 1.5–23.3], $P=0.010$); whereas conventional early EPCs ($CD34^+CD133^+KDR^+$ [>75th percentile]: OR=0.3 [95% CI 0.1–1.0], $P=0.053$) was an independent predictor for reduced risk of coronary calcification at marginal statistical significance.

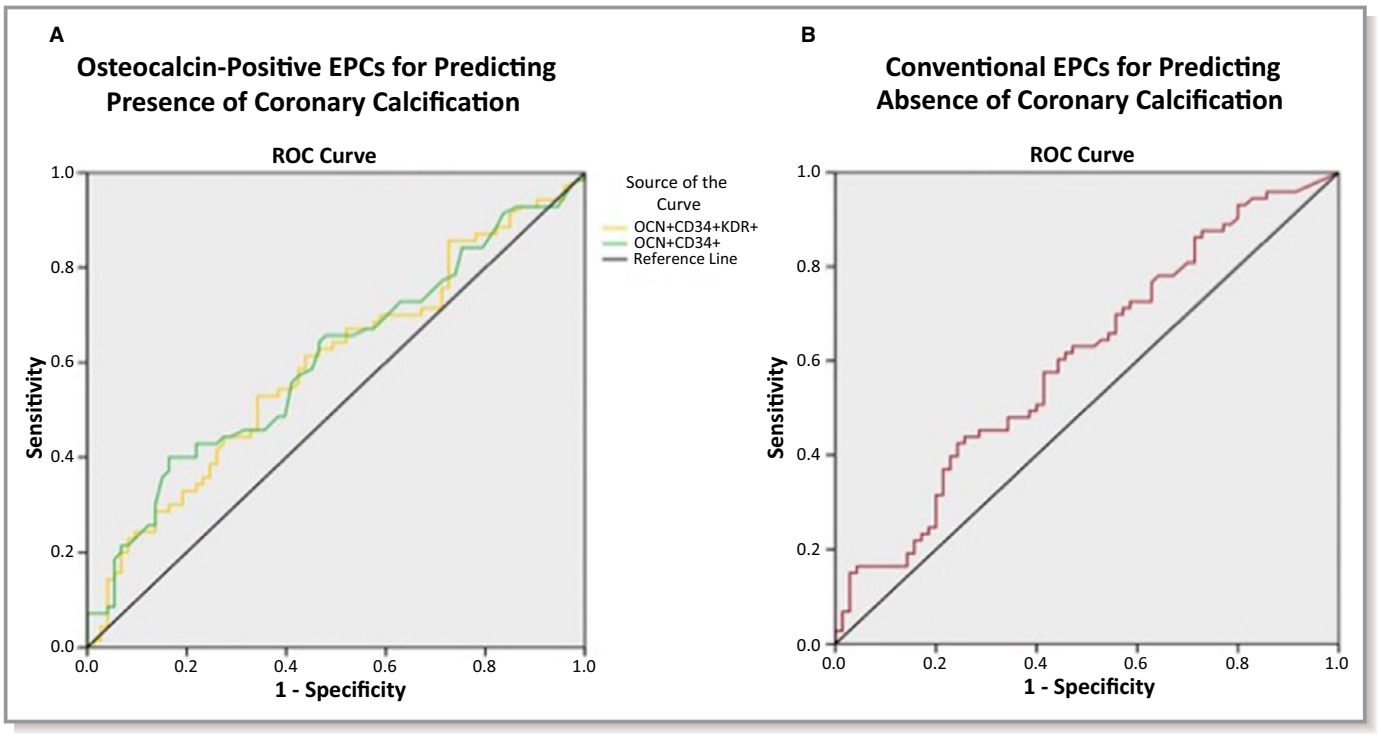

Figure 1. Receiver operating characteristics (ROC) curve analyses revealed divergent behavior of osteocalcin (OCN)-expressing circulating endothelial progenitor cells (EPCs, measured in %) (OCN^+CD34^+ EPCs: area-under-the-curve [AUC]=0.60, $P=0.034$); $OCN^+CD34^+KDR^+$ EPCs: AUC=0.59, $P=0.053$, positive predictor, (**A**) vs conventional early EPCs ($CD34^+CD133^+KDR^+$: AUC=0.60, $P=0.034$, negative predictor, (**B**) in the risk prediction for coronary calcification development.

Table 2. Univariate and Multivariate Predictors for Coronary Calcification*

	Crude Model†		Multivariable Model‡	
	OR [95% CI]	*P* Value	OR [95% CI]	*P* Value
Age (y) in tertiles				
Second tertile	1.83 [0.81–4.13]	0.14	2.61 [0.79–8.61]	0.12
Third tertile	5.08 [2.11–12.22]	<0.001§	5.04 [1.18–21.49]	0.029§
Female, n (%)	0.24 [0.08–0.77]	0.016§	0.37 [0.07–2.11]	0.26
Ever smoking, n (%)	1.60 [0.54–4.74]	0.40	2.23 [0.47–10.63]	0.31
Systolic blood pressure, mm Hg	1.03 [1.01–1.04]	0.004§	1.02 [0.98–1.05]	0.349
Diastolic blood pressure, mm Hg	1.02 [0.99–1.05]	0.13	0.99 [0.95–1.04]	0.81
Resting heart rate, beats/min	1.002 [0.97–1.03]	0.90	1.01 [0.96–1.06]	0.83
LDL-cholesterol, mmol/L	1.04 [0.66–1.66]	0.86	1.29 [0.69–2.43]	0.42
HDL-cholesterol, mmol/L	0.57 [0.27–1.22]	0.15	0.96 [0.26–3.49]	0.95
Triglycerides, mmol/L	0.15 [0.87–2.57]	0.15	0.98 [0.40–2.41]	0.97
HbA1c (%)	4.40 [1.79–10.81]	0.001§	4.12 [1.40–12.10]	0.010§
Serum creatinine, μmol/L	1.04 [1.01–1.06]	0.004§	1.01 [0.98–1.05]	0.47
Statin use, n (%)	1.95 [0.90–4.18]	0.09	0.85 [0.27–2.71]	0.78
DMARDs use (0–4), n (%)‖	0.72 [0.50–1.03]	0.08	0.73 [0.42–1.26]	0.26
RA disease duration, mo	1.04 [0.98–1.09]	0.21	1.00 [0.93–1.08]	0.99
Brachial–ankle pulse wave velocity, cm/s	1.002 [1.001–1.003]	<0.001§	1.001 [0.998–1.003]	0.63
Increased circulating EPCs, $\times 10^3$/mL¶				
OCN-positive EPCs				
$OCN^+CD34^+KDR^+$ EPCs	3.50 [1.53–8.00]	0.003§	7.16 [1.83–27.92]	0.005§
OCN^+CD34^+ EPCs	2.94 [1.31–6.61]	0.009§	5.97 [1.53–23.27]	0.010§
Conventional early EPCs				
$CD34^+CD133^+KDR^+$ EPCs	0.50 [0.23–1.08]	0.08	0.33 [0.11–1.02]	0.053

DMARDs indicates disease-modifying antirheumatic agents; EPCs, endothelial progenitor cells; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; OCN, osteocalcin; RA, rheumatoid arthritis.

*Odd ratio (OR) estimates and 95% CI of coronary calcification explained by variable of interest as estimated by univariable and multivariable logistic regression;

†Unadjusted estimates.

‡Adjusted for potential confounders including age, sex, history of smoking, systolic and diastolic blood pressure, resting heart rate, fasting levels of LDL-/HDL-cholesterol, triglycerides, HbA1c, arterial stiffness, creatinine, use of statins, and DMARDs and duration of RA.

§$P<0.05$.

‖Number of DMARDS used, including hydroxychloroquine, methotrexate, sulfasalazine, and/or leflunomide.

¶Increased circulating EPCs defined as >75th percentile.

Impact of Cumulative Rheumatic Inflammation on Coronary Calcification

Coronary calcification was associated with higher serum levels of CRP (P=0.027) and erythrocyte sedimentation rate (P=0.035). Use of sulfasalazine was associated with reduced risk of coronary calcification (P=0.021). CRI score representing the preceding 60 months of inflammation load was associated with coronary calcification (P=0.004). Receiver-operating characteristics curve analyses showed that CRI score predicted increased risk of coronary calcification (AUC=0.62, P=0.01, Figure 2A), and it remained an independent predictor for coronary calcification after multivariable adjustment for above-stated potential confounders (highest versus lowest quartile: OR=5.6 [95% CI 1.1–28.9], P=0.041, Figure 2B).

Inflammation Affecting EPCs Phenotypes

As shown in Figure 3, CRI score was significantly associated with increased $OCN^+CD34^+KDR^+$ (P=0.016) and OCN^+CD34^+ EPCs (P=0.045), but reduced conventional early $CD34^+CD133^+KDR^+$ EPCs (P=0.049). Multivariable linear regression analyses revealed that CRI remained an independent predictor for increased circulating OCN^+CD34^+ EPCs (highest versus lowest quartile: B=+25.6 [95% CI 0.8–50.5]

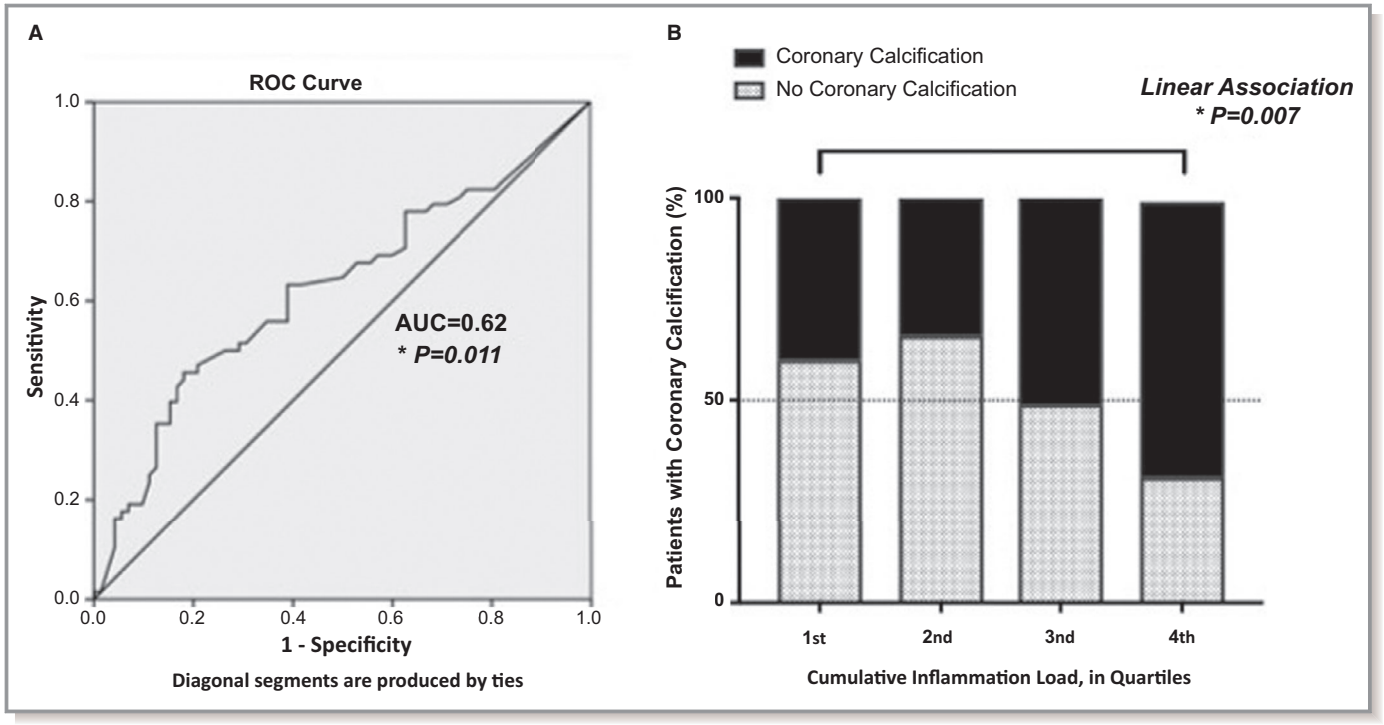

Figure 2. **A**, Cumulative rheumatic inflammation (CRI) score representing preceding 60 months of inflammation load predicted increased risk of coronary calcification (area-under-the-curve=0.62, *P*=0.01). **B**, Increased cumulative rheumatic inflammation (CRI) by quartiles was associated positively with increased risk of coronary calcification (first quartile as reference; second quartile: odds ratio [OR]=0.8 [95% CI 0.3–2.1], *P*=0.62; third quartile: OR=1.6 [95% CI 0.6–4.1], *P*=0.34; fourth quartile: OR=3.3 [95% CI 1.2–8.7], *P*=0.018; overall linear association *P*=0.007). AUC indicates area-under-the-curve; ROC, receiver operating characteristics.

[unit in $\times 10^3$/mL peripheral blood], *P*=0.043, Table S1), but conversely reduced circulating level of conventional early $CD34^+CD133^+KDR^+$ EPCs (highest versus lowest quartile: B=−16.2 [95% CI −31.5 to −0.9] [unit in $\times 10^3$/mL peripheral blood], *P*=0.038, Table S2).

Discussion

To the best of our knowledge, this study was the first to investigate the mechanistic role of OCN expression among circulating EPCs in rheumatic inflammation–promulgated coronary calcification development. Secondly, this was also the first study to show that cumulative inflammation load had an impact on altering circulating EPCs phenotype and their OCN expression, which in turn affects coronary calcification risk in inflammation-prone subjects, exemplified in the study population of RA.

Prior studies showed that patients with RA had differential expression of osteoprotegerin/receptor activator of nuclear factor κ-B (RANK)/ligand (RANKL),[13] a key regulatory system of bone turnover, which may explain at least in part their abnormal rates of osteoporosis and bone erosion.[21] Although the increased risk of cardiovascular events associated with RA has been well described and is independent of conventional risk factors, the exact explanatory mechanisms leading to worsened vascular calcification remained incompletely understood.[22]

Here we established that cumulative rheumatic inflammation load is associated independently with CT-detected coronary calcification. We further showed, through head-on comparisons, that osteogenic circulating $OCN^+CD34^+KDR^+$ and OCN^+CD34^+ EPCs versus conventional early $CD34^+CD133^+KDR^+$ EPCs had essentially divergent pathophysiological roles (ie, detrimental versus protective, respectively) in coronary calcification development. Furthermore, cumulative rheumatic inflammation is associated with altered OCN expression in circulating EPCs. Taken together, these findings may provide the missing mechanistic links to explain the promulgated coronary calcification in RA, as a consequence of inflammation-driven recapitulation of osteoblastic influence on the vasculature.[2]

Indeed, our findings add to and are largely consistent with prior literature. Gossl et al showed that coronary retention of osteogenic EPCs was associated with both the presence and degree of coronary endothelial dysfunction, which was further associated with net production of the inflammatory cytokine interleukin-8.[9] The same study group also showed that patients with severe aortic stenosis had

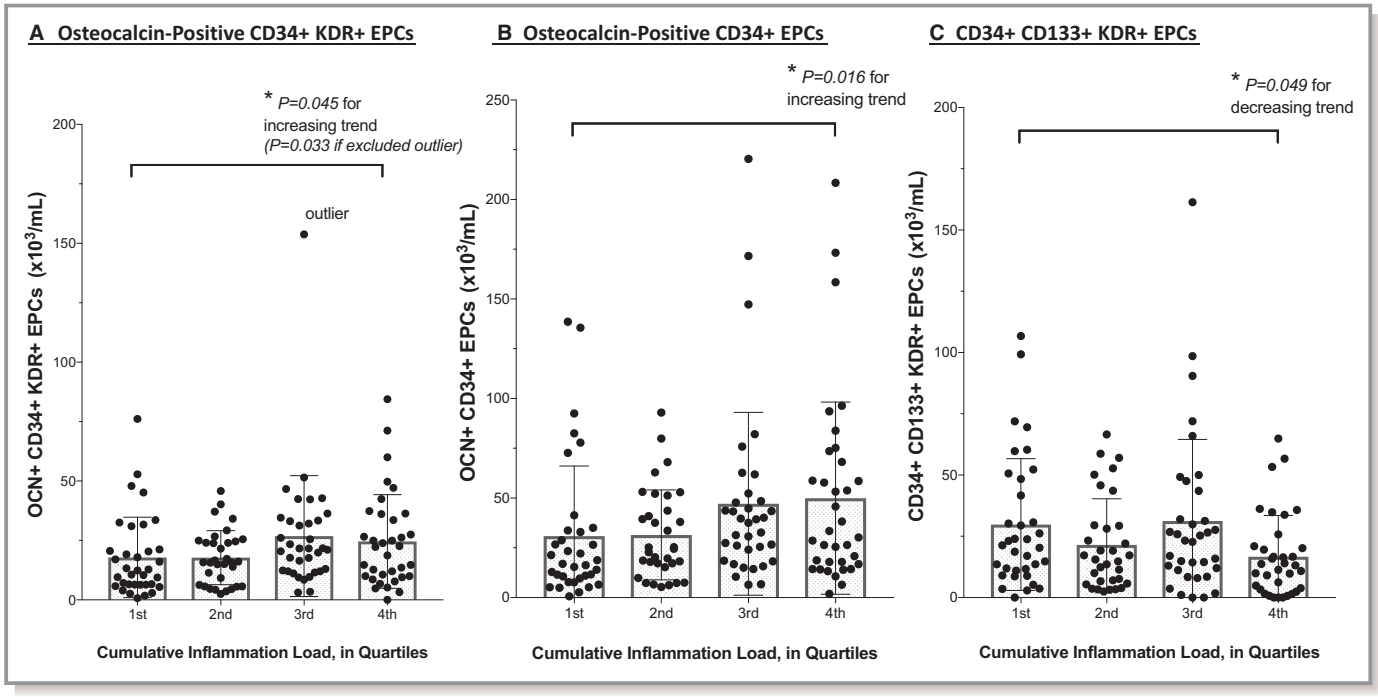

Figure 3. Cumulative rheumatic inflammation (CRI) score was significantly associated with increased osteocalcin-positive (OCN^+) $CD34^+KDR^+$ endothelial progenitor cells (EPCs) (**A**, First quartile: 17.9±16.9; second quartile: 17.8±11.4; third quartile 26.8±25.4; fourth quartile 24.6±19.7; linear upward trend [unweighted] P=0.045, unit in $\times 10^3$/mL peripheral blood) and OCN^+CD34^+ EPCs (**B**, First quartile: 30.9±35.2; second quartile: 31.5±22.6; third quartile: 47.1±45.9; fourth quartile: 50.0±48.3; linear upward trend [unweighted] P=0.016, unit in $\times 10^3$/mL peripheral blood), but reduced conventional early $CD34^+CD133^+KDR^+$ EPCs (**C**, First quartile: 29.8±26.9; second quartile: 21.5±18.8; third quartile: 31.3±33.3; fourth quartile: 16.7±16.8; linear downward trend (combined) P=0.049, unit in $\times 10^3$/mL peripheral blood).

significantly higher circulating osteogenic $CD34^+KDR^+OCN^+$ EPCs. Intriguingly, immunofluorescent studies of the excised stenotic and normal valve tissues showed colocalization of OCN and pro-inflammatory marker nuclear factor κ-B,[15] providing evidence that the bone–vascular axis has widespread influence entailing the whole vasculature.[23] Thus, vascular calcification is a highly active programmed process in which biomineralization with collagen deposition and extracellular matrix modeling are also influenced by paracrine signals orchestrated by the bone morphogenetic proteins, such as the wingless-type MMTV integration site family member (Wnt) pathway, leading to ectopic activation of osteogenic morphogens.[4] Extending this theoretical paradigm, modulation of osteoblast and osteogenic EPCs activities may have a potential role for cardiovascular prevention. For instance, in the MESA (Multi-Ethnic Study of Atherosclerosis) study, nitrogen-containing bisphosphonate use was associated with reduced vascular and valvular calcification in elderly women.[24] A small randomized controlled trial of 20 healthy postmenopausal women further showed that risedronate treatment for 4 months resulted in downregulation of the gene responsible for osteoblast proliferation/differentiation, as well as OCN expression in circulating EPCs. Whether bisphosphate may have a role in vascular protection for RA patients may warrant further studies.

Limitations

First, this is a retrospective cohort study in which residual/undetected biases and confounding cannot be fully eliminated. However, we meticulously maximized our capacity to utilize temporality for causal inference by studying comprehensive preceding cumulative rheumatic inflammation through multiple biomarker encounters up to 60 months before CT coronary angiography, thus strengthening internal validity. We also minimized confounding through multivariable analysis and the findings were consistent and coherent with known literature. Acknowledging that the conventional upper limit of normal CRP for clinical risk assessment is 3 mg/L,[25] in this retrospective study the cut-off for CRP was pragmatically adopted at >0.35 mg/dL, which was the currently quantifiable cut-off value for CRP in our institutional laboratory. Therefore, any residual inflammation below this cut-off cannot be evaluated here. Moreover, it is important to note that the predictive estimate of CRI for coronary calcification, albeit statistically significant, was relatively small (AUC=0.62). Our study subjects had multiple comorbidities, and the

majority of them received disease-modifying antirheumatic drugs resulting in generally good disease control and ameliorated inflammatory markers. These factors may have potential impacts on the generalizability of the study estimates. Furthermore, despite RA being a rare disease overall, the sample size remained relatively small, which limits the power for further subgroup/exploratory analyses other than prespecified. An additional large sample or preferably pooled data analysis of patients with RA will help to enhance the study power, and controlled experimental studies will be required for detailed mechanistic studies.

Conclusions

We conclude that preceding 60 months of cumulative rheumatic inflammation is associated with increased risk of coronary calcification and altered OCN expression in circulating EPCs. Inflammation-driven modulation of the bone–vascular axis warrants further definitive experimental mechanistic studies as a potential mediating mechanism of proatherogenic effects of inflammation.

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Disclosures

None.

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