


ORIGINAL RESEARCH

Serum cholesterol loading capacity on macrophages is linked to coronary atherosclerosis and cardiovascular event risk in rheumatoid arthritis

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ABSTRACT

Objectives Cholesterol loading capacity (CLC) describes the ability of serum to deliver cholesterol to cells. It is linked to foam cell formation, a pivotal step in atherosclerotic plaque development. We evaluate the associations of CLC with coronary atherosclerosis presence, burden and cardiovascular risk in patients with rheumatoid arthritis (RA).

Methods Coronary atherosclerosis (any, high-risk low-attenuation plaque and obstructive plaque) was evaluated with CT angiography in 141 patients. Participants were prospectively followed for 6.0±2.4 years and cardiovascular events including cardiac death, myocardial infarction, unstable angina, stroke, claudication, revascularisation and hospitalised heart failure were recorded. CLC was quantified as intracellular cholesterol in human macrophages after incubation with patient serum.

Results CLC was not linked to overall plaque presence or burden after adjustments for atherosclerotic cardiovascular disease (ASCVD) score, statin use and low-density lipoprotein cholesterol. However, CLC associated with presence and numbers of any, low-attenuation and obstructive plaques exclusively in biologic disease-modifying antirheumatic drugs (bDMARD) non-users (*p* for interaction ≤0.018). CLC associated with cardiovascular event risk overall after adjustments for ASCVD and number of segments with plaque (HR=1.76 (95% CI 1.16 to 2.67) per 1 SD increase in CLC, *p*=0.008). Additionally, bDMARD use modified the impact of CLC on event risk; CLC associated with events in bDMARD non-users (HR=2.52 (95% CI 1.36 to 4.65) per 1SD increase in CLC, *p*=0.003) but not users.

Conclusion CLC was linked to long-term cardiovascular event risk in RA and associated with high-risk low attenuation and obstructive coronary plaque presence and burden in bDMARD non-users. Its prospective validation as a predictive biomarker may be, therefore, warranted.

Excess cholesterol accumulation in arterial wall macrophages and transition to foam cells underlie atherosclerotic plaque formation and growth.¹ Low-density lipoprotein (LDL)

WHAT IS ALREADY KNOWN ABOUT THIS SUBJECT?

- ⇒ Excessive cholesterol accumulation in arterial macrophages and foam cell formation underlie atherosclerotic plaque formation and growth.
- ⇒ Cholesterol loading capacity (CLC) is the ability of serum to deliver cholesterol to cells and relates to foam cell formation. It is increased in conditions associated with cardiovascular risk, including rheumatoid arthritis (RA).

WHAT DOES THIS STUDY ADD?

- ⇒ Serum CLC was linked to long-term cardiovascular risk in patients with RA, even after controlling for atherosclerotic cardiovascular disease risk score and plaque burden. Biologic disease-modifying antirheumatic drugs (bDMARD) use moderated the effect of CLC on cardiovascular risk. CLC associated with increased cardiovascular risk exclusively in bDMARD non-users.
- ⇒ CLC associated with coronary plaque presence and burden in bDMARD non-users.

HOW MIGHT THIS IMPACT ON CLINICAL PRACTICE OR FUTURE DEVELOPMENTS?

- ⇒ CLC may serve as an independent, predictive cardiovascular risk biomarker in both RA as well as general patients on prospective validation in larger studies.

is the principal transporter of cholesterol in the serum. Normally, the ability of LDL to deliver cholesterol to cells by LDL is regulated by intracellular cholesterol content; increasing concentration of intracellular cholesterol attenuates surface expression of LDL receptors.² However, in the context of systemic inflammation or increased oxidative stress, changes in LDL structure enable cholesterol loading—especially to vessel wall macrophages—through alternative and

unregulated pathways.³ Cholesterol loading capacity (CLC) is a recently described parameter reflecting the ability of serum to supply cholesterol to human macrophages cultured in standard conditions. CLC measurement is simple and involves the quantification of cholesterol in cell lysates after treatment with serum.⁴ CLC is linked to foam cell formation⁵ and is increased in conditions associated with higher cardiovascular risk.^{6–8} These cholesterol-loaded foam cells are generally considered proatherogenic⁷ while a recent report suggested that they might be atheroprotective.^{9–10} Ultimately, the associations between CLC, subclinical atherosclerosis and cardiovascular event risk have not been reported.⁷

Serum from patients with rheumatoid arthritis (RA) increased macrophage cholesterol uptake and foam cell formation more compared with serum from controls.⁵ Likewise, patients with RA display greater coronary atherosclerosis burden, higher risk plaque features and cardiovascular event rates than age-matched and gender-matched non-RA controls.^{11–12} In contrast, biologic therapies associated with lower cardiovascular risk,^{13–16} decreased formation of new coronary plaques and enhanced stabilisation of prevalent atherosclerotic lesions.¹³ Importantly, the cardioprotective benefit of RA treatments beyond their anti-inflammatory properties may reflect targeted effects on macrophage cholesterol homeostasis, which may include decrease in CLC.^{4,17}

Our main objective was to evaluate the relationships of CLC with coronary atherosclerosis and cardiovascular event risk in patients with RA. A secondary aim was to explore potential moderating effects of biologic therapies on these relationships.

MATERIALS AND METHODS

Patient recruitment

One hundred and forty-one patients with RA participating in the PROspecTive Evaluation of Latent Coronary ATtherosclerosis in RheumatoidArthritis (PROTECT-RA) cohort¹¹ and serum available for CLC assessments were evaluated. The main cohort was comprised of 150 patients with RA from a single centre who had CT angiography (CCTA) evaluation of coronary plaque from March 2010 to 2011. Patients were prospectively followed for incident cardiovascular events over 6.0±2.4 years. Subjects were 18 to 75 years old, satisfied 2010 classification criteria for RA and had no known cardiovascular disease such as angina, myocardial infarction, stroke, transient ischaemic attack, claudication, revascularisation or heart failure. Subjects were excluded if they had concomitant autoimmune syndromes (other than Sjogren's), body weight exceeding 147.7 kg, active malignancy within 5 years, active or chronic infections, allergy to iodine or glomerular filtration rate <60 mL/min. The local Institutional Review Board approved this study and patients signed informed consent according to the Declaration of Helsinki.

Coronary CT angiography

Coronary CT angiography (CCTA) evaluations were carried out in a 64-multidetector row scanner. CCTA image acquisition and processing protocols¹⁸ and scoring reproducibility¹⁹ were formerly reported. Studies were evaluated by a singular experienced interpreter (MJB) who was blinded to patients' clinical information. The Agatston method was used to evaluate coronary artery calcium (CAC) score.²⁰ Coronary atherosclerosis was assessed on contrast-enhanced scans according to a formal 17-segment American Heart Association model.¹⁹ The total number of segments harbouring plaque per patients was reported as segment involvement score (0–17). Atherosclerotic plaques rendering >50% of the vessel lumen were reported as obstructive. Plaque composition was studied and atherosclerotic lesions were further evaluated for low attenuation areas (≤30 Hounsfield units), which correspond to necrotic lipid cores and considered high-risk features for plaque rupture.²¹

Serum CLC

Fasting blood samples were collected the day of CCTA and serum aliquots were frozen to –80°C until assayed in batches as previously described.²² CLC was quantified as intracellular cholesterol content in human leukemia cell line THP-1 monocyte-derived macrophages using a fluorimetric assay.⁴ Specifically, THP-1 cells were cultured with 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 72 hours to allow differentiation into macrophages. Individual patient sera were subsequently added to the culture at 5% dilution for 24 hours. Next, the cells were washed and lysed, and a fluorimetric assay was used to measure cholesterol content in cell lysates. CLC values were reported as micrograms of cholesterol per milligram of protein.

Covariates and outcome definitions

The 10-year atherosclerotic cardiovascular disease (ASCVD) risk score based on the pooled cohort equation was calculated for all participants at baseline.²³ Disease activity and medication use were recorded at baseline and all subsequent clinic visits (every 3 to 4 months) during follow-up. Disease activity was estimated based on a standard 28-joint examination for tenderness and swelling and C reactive protein. Medications recorded included conventional synthetic disease-modifying antirheumatic drugs (csDMARDs), biologic DMARDs (bDMARDs), prednisone and statins.

The composite cardiovascular event outcome including cardiac death, myocardial infarction, unstable angina, stroke, transient ischaemic attack, peripheral arterial disease and heart failure was prespecified as the clinical outcome of interest. All events were confirmed by review of electronic medical records and adjudicated by the respective specialists using standard definitions.²⁴ Adjudicators were blinded to prior CCTA results. Analyses considered only first cardiovascular events.

Coronary atherosclerosis outcomes of interest included CAC score and the presence (per-segment) and number (per-patient) of any, low-attenuation and obstructive plaques.

Statistical analysis

Continuous variables were reported as means with SD and categorical variables as frequencies with percentages. The effect of CLC on the likelihood of per-segment plaque presence was evaluated with robust logistic regression adjusting for proximal segment location and using a robust cluster-adjusted sandwich estimator to account for correlation of coronary segments within patients. In per-patient analyses, robust negative binomial regression was used for count outcomes (number of plaques) and linear regression for CAC. CAC had a skewed distribution so was analysed as natural log transformation (ln) of (CAC+1) because 66% of patients had zero CAC.²⁵ Effect modification by baseline bDMARD use was evaluated by adding bDMARD use and a CLC × bDMARD interaction term to the models. Each multivariable plaque outcome model adjusted for ASCVD risk score, statin use and LDL cholesterol (LDL-C). The impact of CLC on incident cardiovascular event risk was evaluated in a Cox regression model adjusting for ASCVD risk score and segment involvement score. Additionally, a Cox regression model with CLC, baseline bDMARD use and a CLC × bDMARD interaction term as predictors assessed whether bDMARD use moderated the effect of CLC on cardiovascular event risk. SPSS V.27 and Stata V.15 were used. P values < 0.05 were considered significant.

RESULTS

Patients were primarily middle-aged women with long-standing, seropositive disease (table 1). All participants were treated with csDMARDs and 86/141 (61%) additionally received bDMARDs (all Tumor necrosis factor- α [TNF- α] inhibitors) on enrolment. General cardiac risk factors, ASCVD risk scores, disease activity, DMARD therapies and quantitative coronary plaque outcomes were similar between bDMARD users and non-users (table 1). Frequencies of various per-patient plaque types are reported in online supplemental table 1.

Associations of CLC with coronary atherosclerosis presence and burden

Mean (SD) serum CLC was 12.70 (2.85) μ g cholesterol/mg protein and was similar in bDMARD users and non-users. There was no main effect of CLC on any per-segment (figure 1) or per-patient plaque outcome (figure 2). However, bDMARD use significantly moderated the effect of CLC on several plaque outcomes. Specifically, higher CLC associated with an increased likelihood of per-segment presence of low attenuation and obstructive plaque in patients with bDMARDs non-users but not those treated with bDMARDs (figure 1). For per-patient plaque outcomes, CLC predicted a higher number of segments with any plaque, low-attenuation plaque and

obstructive plaque only in bDMARD non-users but not bDMARD-treated patients (figure 2 and figure 3). While CLC was not associated with ln(CAC +1) adjusting for ASCVD risk score, statin use and LDL-C (β per 1 SD higher CLC, 0.05 (95% CI -0.13 to 0.13), p=0.539), the effect of the CLC × bDMARD interaction on ln(CAC+1) was significant (p-for-interaction=0.014, figure 3); CLC associated with ln(CAC+1) in bDMARD non-users (β per 1 SD higher CLC, 0.25 (95% CI 0.04 to 0.46), p=0.022) but not those treated with bDMARDs (β per 1 SD higher CLC, -0.07 (95% CI -0.24 to 0.09), p=0.372).

CLC is linked to greater risk of cardiovascular events

Over 6.03±2.42 years of follow-up, 15 patients experienced 18 cardiovascular events (2.08 (95% CI 1.31 to 3.30) events/100 patient-years (online supplemental table S2). In a multivariable Cox regression model, higher CLC associated with greater cardiovascular event risk (HR per 1 SD higher CLC, 1.76 (95% CI 1.16 to 2.67), p=0.008, figure 4A) after controlling for ASCVD risk and segment involvement score. Notably, bDMARD use moderated the effect of CLC on event risk (p for interaction=0.009). In a separate Cox regression model with CLC, baseline bDMARD use and a CLC × bDMARD interaction term as predictors, CLC associated with higher cardiovascular event risk in bDMARD non-users (HR per 1 SD higher CLC, 2.52 (95% CI 1.36 to 4.65), p=0.003, figure 4B) but not in bDMARD users (HR per 1 SD higher CLC, 0.85 (95% CI 0.49 to 1.49), p=0.574).

DISCUSSION

CLC is an emerging measure of lipoprotein functions related to cell cholesterol regulation and associates with foam cell formation.⁵ It is a simple parameter, quantifying human macrophage cholesterol content after incubation with patient serum in standard conditions. Recent studies, all applying the same method of measurement, report that CLC is increased in conditions associated with high cardiovascular risk, but its connection with atherosclerosis has never been explored. This is the first study to formally interrogate the link between CLC and coronary atherosclerosis and cardiovascular event risk. Despite the absence of a main effect of CLC on atherosclerosis in the total sample, treatment with TNF inhibitors (TNFi) moderated the relationship between CLC and plaque presence and burden. Specifically, higher CLC was linked to greater likelihood of low attenuation and obstructive plaque presence in coronary segments of TNFi non-users, but not in TNFi users. Moreover, higher CLC predicted a higher CAC score and greater number of coronary segments with any plaque, low attenuation plaque and obstructive plaque in TNFi non-users but not in TNFi users.

Inflammatory monocyte recruitment to atherosclerotic lesions and their local differentiation to macrophages promotes atherosclerosis by increasing lesion cellularity.^{26 27} Cholesterol loading of macrophages in plaque

Table 1 Baseline characteristics

	Total sample (n=141)	No bDMARD (n=55)	bDMARD (n=86)
Age (years)	52.95±10.50	52.84±10.90	53.03±10.30
Female	124 (88)	50 (91)	74 (86)
RA duration (years)	10.50±7.64	8.61±7.16	11.71±7.73
Age at diagnosis	42.45±11.20	44.22±11.13	41.31±11.17
RF positive	121 (86)	46 (84)	75 (87)
ACPA positive	120 (85)	41 (75)	79 (92)
Erosions	93 (66)	35 (64)	58 (67)
Swollen joint count	1.70±2.62	1.67±2.44	1.72±2.75
Tender joint count	1.38±2.64	1.31±2.58	1.43±2.69
CRP mg/L	8.61±12.52	8.50±11.49	8.68±13.20
DAS28-CRP	2.54±1.00	2.51±0.94	2.56±1.04
Cholesterol (mmol/L)	4.38±0.91	4.53±0.94	4.28±0.88
LDL-C (mmol/L)	2.46±0.73	2.58±0.78	2.38±0.69
HDL-C (mmol/L)	1.33±0.36	1.36±0.37	1.31±0.36
Hypertension	65 (46)	24 (44)	41 (48)
Systolic BP (mm Hg)	128.28±15.31	125.67±16.69	129.95±14.21
Diastolic BP (mm Hg)	73.17±8.88	72.65±9.87	73.50±8.22
Diabetes	23 (16)	9 (16)	14 (16)
Current smoking	12 (9)	3 (5)	9 (10)
Body mass index (kg/m ²)	28.94±5.58	27.59±5.21	29.81±5.67
ASCVD risk score	4.96±6.81	4.31±5.33	5.37±7.60
Statin use	53 (38)	20 (36)	33 (38)
Prednisone use	48 (34)	13 (24)	35 (41)
Methotrexate use	113 (80)	45 (82)	68 (79)
No. concurrent csDMARDs	1.94±0.79	1.91±0.75	1.95±0.82
Plaque presence (any)	99 (70)	37 (67)	62 (72)
No. plaques	2.00±2.32	1.93±2.23	2.05±2.38
No. obstructive plaques	0.26±0.86	0.15±0.56	0.33±1.00
No. low-attenuation plaques	0.30±0.78	0.25±0.58	0.34±0.89
CLC (mg chol/mg protein)	12.70±2.85	12.60±2.72	12.76±2.94

Values are mean±SD or n (%). There are no missing data for any of the predictors or outcomes reported.

ACPA, anti-cyclic citrullinated peptide antibodies; ASCVD, atherosclerotic cardiovascular disease score; bDMARD, biologic disease modifying anti-rheumatic drug; BP, blood pressure; CLC, cholesterol loading capacity; csDMARDs, conventional synthetic disease modifying anti-rheumatic drugs; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RF, rheumatoid factor.

stimulates cytokine secretion, endoplasmic reticulum stress and apoptosis.^{28,29} In early atherogenesis, apoptotic cell clearance by activated phagocytes (efferocytosis) associates with a reduction in the lesion cellularity and decreased atherosclerosis progression.³⁰ Treatment of atherosclerosis-prone mice with adalimumab, resulted in deposition of the fluorescent-tagged drug was deposited in atherosclerotic lesions where it suppressed influx of monocytes into the arterial wall and inhibited TNF release from macrophages.³⁰ Accordingly, we previously reported that bDMARD treatment predicted decreased new plaque formation in patients with RA without coronary atherosclerosis or with early non-calcified lesions.¹³

In later stages of atherosclerosis, inefficient efferocytosis leads to increased inflammation and formation of necrotic lipid core depicted as low attenuation lesions on CCTA.³¹ Treatment with TNFi was shown to restore efferocytosis through macrophage LDL receptor-related protein 1—a recognition receptor for apoptotic cell removal—and, therefore, reduce necrosis and inflammation in plaques of atherosclerosis-prone mice.³² It is, therefore, possible that, even in the face of increased CLC, apoptotic foam cells would be more effectively cleared from atherosclerotic lesions in TNFi-treated patients, attenuating plaque progression inflammation and instability. Correspondingly, we showed that bDMARD therapy was linked to loss

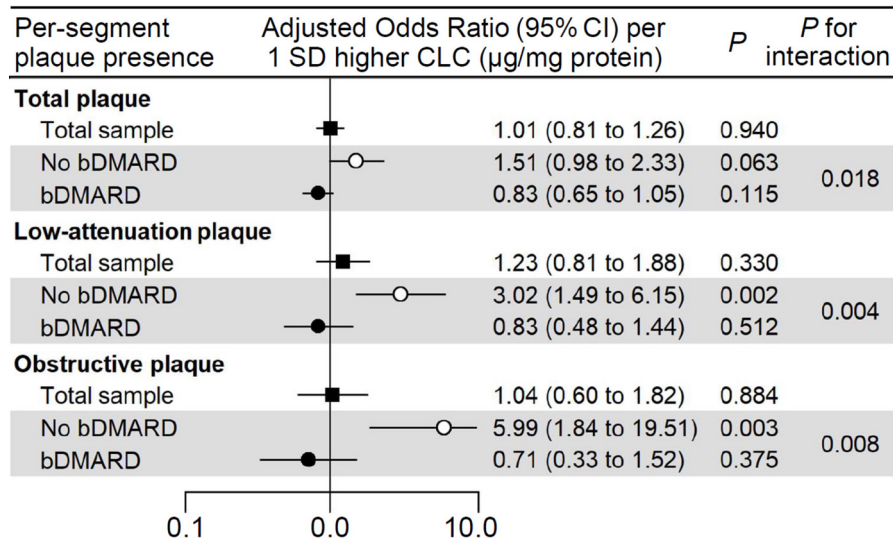


Figure 1 Association of serum CLC with per-segment coronary plaque outcomes for the total sample and stratified by bDMARD use. ORs derived from robust binary logistic regression adjusted for proximal segment location, atherosclerotic cardiovascular disease (ASCVD) score, statin use and LDL-C. bDMARD, biologic disease modifying anti-rheumatic drugs; CLC, cholesterol loading capacity; LDL-C, low-density lipoprotein cholesterol.

of low attenuation cores from prevalent atherosclerotic lesions and formation of more stable fibrous or fibrocalcific plaques.¹³ Additionally, treatment of patients with RA with adalimumab significantly lowered macrophage cholesterol uptake independently of its ability to inhibit soluble TNF.⁴ In fact, adalimumab was shown to engage surface TNF α expressed on live human macrophages and was quickly internalised after priming with lipopolysaccharide (LPS).⁴ This event is associated with reverse signalling culminating in lower macrophage cholesterol uptake from both normal as well as hypercholesterolemic serum.⁴ Thus, even in the presence of circulating lipoproteins with increased potential to deliver cholesterol

to cells (reflected by the CLC parameter), adalimumab might inhibit actual macrophage cholesterol uptake by binding to membrane-expressed TNF. Thus, the uncoupling of CLC with atherosclerosis might be also explained by this direct effect of adalimumab on the atherosclerotic plaque macrophages expressing membrane TNF.

We here report that baseline CLC was linked to incident cardiovascular event risk in the entire sample after accounting for both clinical risk score and coronary atherosclerosis. Therefore, CLC may further influence cardiovascular risk through effects on atherosclerotic plaque biology, independently of quantitative anatomic and morphologic characteristics, that may include

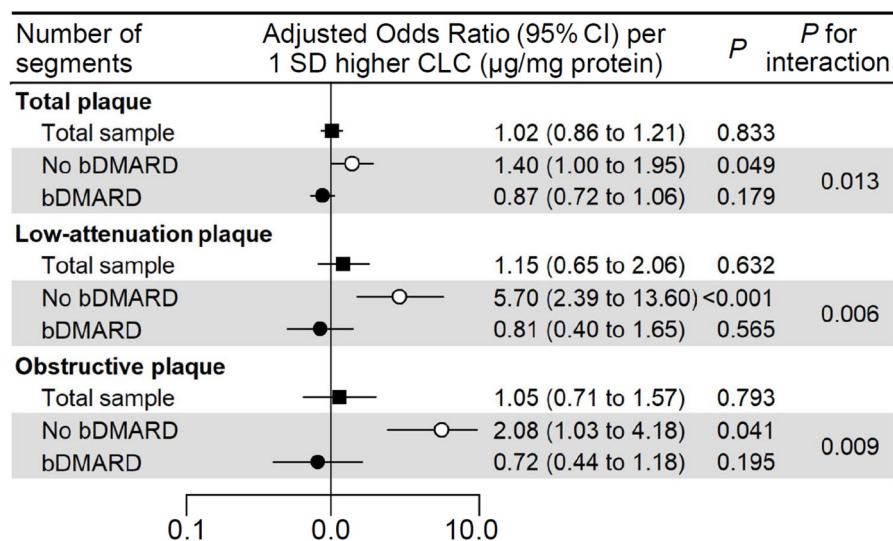


Figure 2 Association of serum CLC with per-patient coronary plaque outcomes for the total sample and stratified by bDMARD use. Rate ratios denote the per cent change in number of segments with plaque associated with one SD unit increase in CLC. Rate ratios derived from multivariable negative binomial regression models adjusted for atherosclerotic cardiovascular disease (ASCVD) score, statin use and LDL-C. bDMARD, biologic disease modifying anti-rheumatic drugs; CLC, cholesterol loading capacity; LDL-C, low-density lipoprotein cholesterol.

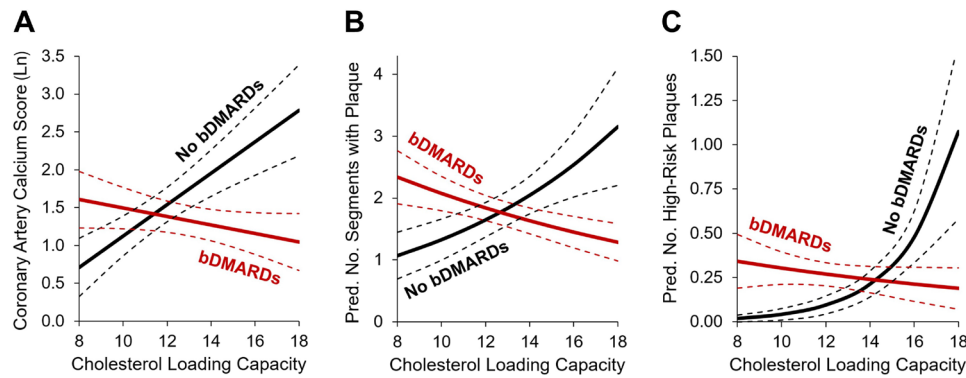


Figure 3 Coronary plaque burden outcomes across levels of serum CLC stratified by bDMARD use. (A) Coronary artery Calcium score. (B) Predicted number of segments with plaque per patient. (C) Predicted number of high-risk plaque per patient. Dashed lines represent standard errors. bDMARD, biologic disease modifying anti-rheumatic drugs; CLC, cholesterol loading capacity.

influencing the function of plaque resident cells and also cells that interact with atherosclerotic lesions on the luminal side such as platelets and erythrocytes; these cells also participate in thrombus formation once a plaque ruptures or erodes.³³ Specifically, oxidised LDL may load on vascular smooth muscle cells via lectin-like oxidised LDL-receptor type-1 (LOX-1) and promote apoptosis, leading to atherosclerotic plaque instability and rupture.³⁴ Cholesterol loading onto platelets increases their activation³⁵; oxidised LDL can load onto platelets through surface CD36 or LOX-1 inducing platelet activation, enhanced adhesion to endothelial cells and adenosine diphosphate (ADP)-mediated aggregation, all of which favour thrombus formation.^{34 36 37} Accumulation of cholesterol in platelet membranes may modify the structure of lipid rafts containing surface receptors and promote signalling that potentially fosters atherothrombosis.^{38 39} In fact, membrane cholesterol content on platelets and erythrocytes was reported as a risk factor for cardiovascular events.^{40 41}

Beyond an overall association with cardiovascular risk, the relationship of CLC with incident events was further-moderated by bDMARD use. CLC associated with cardiovascular risk exclusively in bDMARD nonusers.

Our study has certain limitations. First, CLC is the result of the activity of circulating lipoproteins both delivering and accepting cholesterol to and from cells. Although LDL modification is considered a main determinant of CLC, and in our experimental conditions, the impact of cell cholesterol efflux is likely very limited, the potential influence of lipoproteins other than LDL deserves a specific study. Second, our original study design was not powered to specifically evaluate the relationships between CLC and coronary atherosclerosis presence, burden or cardiovascular events; hence, these findings are exploratory. Third, since this is the first study examining the relationships between CLC, coronary atherosclerosis and cardiovascular events in any clinical state, our findings may not be generalisable to non-RA patients. Moreover, most study participants self-identified as Hispanic whites,

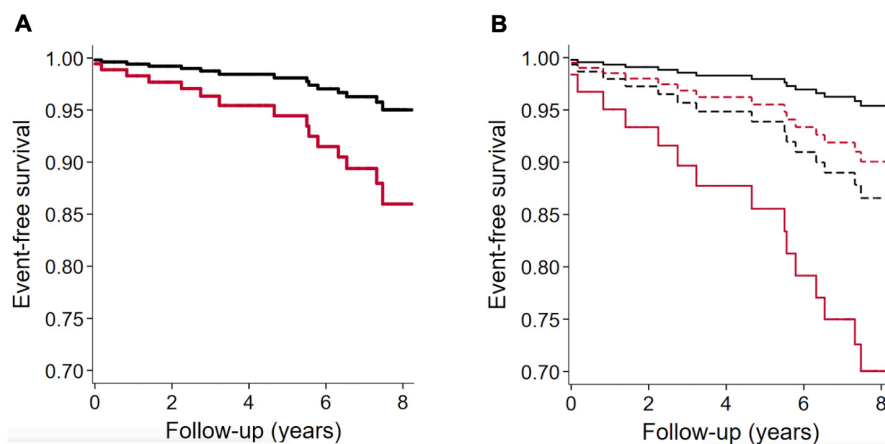


Figure 4 Association of serum CLC with cardiovascular event risk. (A) Higher CLC associates with greater cardiovascular risk after adjustments for ASCVD risk score and number of coronary segments with plaque. Red line represents high serum CLC (+1 SD) and black line represents low serum CLC (-1SD). (B) CLC associated with higher cardiovascular event risk in bDMARD nonusers but not in bDMARD users. Solid lines represent bDMARD nonusers and dashed lines represent bDMARD users. Red lines represent high serum CLC (+1 SD) and black lines represent low serum CLC (-1 SD). ASCVD, atherosclerotic cardiovascular disease; bDMARD, biologic disease modifying anti-rheumatic drugs; CLC, cholesterol loading capacity.

which may also limit the generalisability of our findings. Finally, causal relationships cannot be inferred since this is an observational study and use of medications was non-randomised.

Conclusion

Serum CLC was linked to long-term cardiovascular event risk even after controlling for ASCVD risk score and plaque burden in the entire sample of patients with RA. Prospective validation of CLC as an independent, predictive cardiovascular risk biomarker in both RA as well as general patients may be, therefore, warranted. Additionally, the influence of CLC on cardiovascular risk was moderated by bDMARD use; CLC predicted greater cardiovascular risk in bDMARD non-users. Moreover, CLC associated with coronary plaque presence, burden and high-risk composition in bDMARD non-users. These data expand our understanding of the mechanisms underlying the cardioprotective effects of bDMARDs in RA.

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Contributors GAK and NR conceived and designed the study. GAK, SRO, EH and MJB collected the data. GAK, BP, SRO, MP, EH, MPA, FZ, MJB and NR analysed and interpreted the data. All authors were involved in development, review and approval of the manuscript. GAK is the overall content and contribution guarantor, has full access to study data and is accountable for the accuracy and integrity of the work.

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Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by John F Wolf Human Subjects Committee at the Lundquist Institute and Harbor-UCLA Medical Center IRB number: 18CR-22637-01.

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