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RESEARCH ARTICLE

Rosuvastatin Improves Endothelial Progenitor Cells in Rheumatoid Arthritis

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Abstract

Background: Endothelial Progenitor Cells (EPCs) are depleted and contribute to increased cardiovascular (CV) risk in rheumatoid arthritis (RA). Statins exert a protective effect in CAD partly by promoting EPC mobilization. This vasculoprotective effect of statin has not yet been investigated in RA. We aimed to investigate the effect of rosuvastatin on EPCs in RA.

Methods:
50 RA patients were randomized to receive 6 months of treatment with rosuvastatin (10mg/day, n=25) and placebo (n=25) as an adjunct to existing stable antirheumatic drugs. EPCs ($CD34^+/CD133^+$) were quantified by Flow Cytometry. Inflammatory measures included DAS28, CRP and ESR were measured at baseline and after treatment. Lipids and pro-inflammatory cytokines (TNF-α, IL-6 and IL-1) were estimated at baseline and after treatment.

Results:
At baseline, inflammatory measures and pro-inflammatory cytokines were elevated and EPCs depleted among both groups. At baseline, EPCs inversely correlated with DAS28 and TNF-α in both groups. EPCs increased significantly ($p<0.01$) after treatment with rosuvastatin but did not show significant change with placebo. Rosuvastatin exerted positive effect on lipid spectrum: lowering total cholesterol, LDL, non HDL and elevation of HDL as compared with placebo. At 6 months, DAS28, ESR, CRP, TNF-α and IL-6 improved significantly in rosuvastatin group. Significant negative correlation was observed between EPCs and DAS28, CRP, TNF-α and IL-6 after treatment with rosuvastatin.

Conclusion:
First study to show that rosuvastatin improves inflammation and EPC biology in RA possibly through its anti-inflammatory and lipid lowering effect. This beneficial effect of rosuvastatin may provide a novel strategy to prevent cardiovascular events in RA.

Introduction

Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease characterized by increased cardiovascular morbidity and mortality mainly due to an acceleration of atherosclerotic damage and many disease-related pathogenic mechanisms take part in this process [1, 2]. A large body of evidence supports the involvement of common proinflammatory cytokines in the development and progression of both RA and atherosclerosis. The destructive proinflammatory cascade and effector mechanisms implicated in RA resemble the chronic inflammatory

processes that drive the development of atherosclerosis in general. Proinflammatory cytokines such as interleukins-(IL-1, IL-6) and tumor necrosis factor-α (TNF- α) produced locally within affected joints in RA may promote both traditional (e.g., dyslipidemia, insulin resistance) and nontraditional (e.g., oxidative stress) systemic cardiovascular risk factors [3]. The over expression of these pro-inflammatory cytokines in chronic inflammation may thus participate in this increased cardiovascular risk. RA is also associated with enhanced angiogenesis and disturbed vasculogenesis [4]. EPCs have been identified in synovial tissue of rheumatoid arthritis patients where they participate in angiogenesis [5]. Reduced numbers and/or altered functions of EPCs have recently been demonstrated in patients with inflammatory rheumatic disorders like rheumatoid arthritis (RA) [6], systemic sclerosis (SSc) [7] and systemic lupus erythematosus (SLE) [8] associated with increased cardiovascular morbidity and mortality. These problems may limit endogenous repair of vascular lesions, leading to progression of atherosclerosis. Therefore, increasing the number and function of EPCs is necessary for the prevention and/or treatment of vascular diseases such as atherosclerosis. The lifespan of EPCs has been reported to be shortened by higher levels of serum IL-6 and TNF-α and associated with enhanced cardiovascular risk (CV) risk in RA patients [4, 9]. It is therefore possible that inflammation induced by pro-inflammatory cytokines induces endothelial damage by shortening the EPC lifespan and decreasing EPC function. Given the potential link between EPCs and inflammation, we hypothesized that the decrease in circulating EPCs in RA may be due to pro-inflammatory cytokines. Drugs with anti-inflammatory properties, such as statins, have been demonstrated to exert beneficial effects on EPC biology in patients with stable coronary artery disease and diabetes [10, 11] but the effect of statins has not yet been investigated in RA. Hence, we aimed to investigate the effect of rosuvastatin on EPCs in RA patients.

Material and methods

Patients

Fifty RA patients (aged >18 years) who fulfilled the 2010 Rheumatoid Arthritis Classification Criteria for diagnosis and classification of RA were enrolled in the study from a rheumatology outpatient clinic [12]. These patients were randomized to receive 6 months of treatment with rosuvastatin (10mg/day, n=25: 4 male, 21 female; mean age 45±0.92 years, range 23-65) and placebo (n=25: 2 male, 23 female; mean age 46 ± 0.95 years, range 24-67) as an adjunct to existing stable antirheumatic drugs, with active RA, defined by the presence of modified Disease Activity Score (DAS28>3.2). Exclusion criteria included patients with diabetes mellitus, hepatic and renal failure, peripheral artery disease, stroke, coronary artery disease, hypertension and smokers. None of the patients were receiving statins, angiotensin receptor blockers, angiotensin converting enzyme inhibitors, TNF-α inhibitors and nitrates which have been shown to affect the endothelial progenitor cell count, or any other cardiovascular medications. Patients had to be taking stable doses of DMARDs for at least 3 months before entering the study.

The study protocol was approved by the regional ethical research committee and was performed in accordance with the declaration of Helsinki and the code of Good Clinical Practice. All patients provided written informed consent to participate after a full explanation of the study.

Assessment

In all subjects, blood was drawn in the morning after overnight fasting and the following variables were determined: complete blood count, erythrocyte sedimentation rate (ESR), C-reactive protein, lipids and fasting blood sugar by conventional methods using standard commercial kits.

Assessment of Endothelial Progenitor Cells (EPCs) by Flow cytometry:

Flow cytometry analysis: EPCs were quantified by fluorescence-activated cell sorting (FACS) analysis by Calibur flow cytometer (Canto III, BD Biosciences). Three colour analysis was performed using CD45 FITC (BD Sciences), CD34 PE (BD Sciences), and CD133 APC (Miltenyi Biotec) antibodies [13]. Peripheral blood was taken at rest, in the morning, at forearm, together with routine analysis in patients. Two hundred micro-liters of peripheral blood in sodium heparin was labelled with a panel of above mentioned antibodies and incubated for one hour at room temperature. After conjugation, red blood cells were lysed by incubating in FACS lysing solution (BD Bioscience) for 15 minutes at room temperature. Thereafter, cells were washed and resuspended in 500 µl phosphate buffered saline (PBS; Seromed). Appropriate analysis gates were used to enumerate total and activated EPCs and to exclude debris. Cells stained with FITC were used as negative controls. At least 100,000 - 250, 000 cells per sample were acquired. Data were analyzed with CellQuest software (Becton Dickinson). Results are expressed as % cells gated. Investigators involved in EPC estimation were blinded to the treatment protocol.

Assessment of inflammatory disease activity: The following measures of clinical evaluation of inflammation were employed.

Estimation of Pro-inflammatory Cytokines i.e. TNF-α, IL-6 and IL-1 was done by using Standard ELISA kits (Diaclone SAS, France). Disease Activity Score of 28 joints (DAS28) was used to assess disease activity of 28

joints as a composite measure, which is a linear sum of four parameters including Tender joint count, Swollen Joint Count, patient global assessment of general health on a visual analogue scale (VAS). ESR was measured by Westergren method and CRP level was determined using standard commercial kits.

Statistical Analysis

Test values are reported as mean ± SEM. Spearman analysis was used to find the relationship between EPCs and pro-inflammatory cytokines. A p value<0.05 was considered to indicate significant difference. Statistical analysis was done using the Prism Software 5.04 version for Windows 8.0.

Results

Patient profile

The baseline demographic and clinical characteristics of the patients on treatment and placebo are presented in the Table I and the patients characteristics before and after therapy are presented in Table II. The patients included in this study were all adults with established active disease.

Endothelial Progenitor Cells (EPCs)

At baseline, the number of circulating Endothelial Progenitor Cells (EPCs), analyzed by flow cytometry, were significantly decreased in both treatment and placebo group. After 6 months of treatment, EPCs were significantly increased in RA patients treated with rosuvastatin as compared to the baseline values (Table II). Placebo treatment did not affect EPC counts (Table II).

Inflammatory disease activity measures

All patients included in the study had active disease with DAS28 score $\geq$ 3.2. After treatment with rosuvastatin, DAS28 score improved significantly ($p< 0.05$) while there was no significant improvement in DAS28 in placebo group. ESR and CRP level also decreased significantly, after treatment with rosuvastatin as compared to placebo group (Table II).

Inflammatory Cytokines

At baseline, levels of pro-inflammatory cytokines i.e. TNF-α, IL-6, and IL-1 were higher in both groups (Table I) suggesting that higher levels of pro-inflammatory cytokines in active disease are associated with reduced number of EPCs in RA patients. After treatment with rosuvastatin there was a significant decrease in TNF-α and IL-6 as compared with placebo group. However, there was no significant improvement in IL-1 in both groups.

Lipid profile

Lipids levels were altered i.e. increased total cholesterol, LDL cholesterol, non-HDL cholesterol and decreased HDL cholesterol in both treatment and placebo groups at baseline. Treatment with rosuvastatin resulted in significant decrease in total cholesterol, LDL and non-HDL cholesterol and increase in HDL cholesterol as compared to placebo group [Table II].

Association of EPCs with pro-inflammatory cytokines and disease activity measures

At baseline, EPCs were inversely related with DAS 28 and TNF-α in both rosuvastatin and placebo group (Table III).

After treatment with rosuvastatin, EPCs levels correlated inversely with DAS 28 ($r=-0.49$, $p=0.03$), CRP ($r=-0.44$, $p=0.02$), TNF-α ($r=-0.42$, $p=0.04$) (Fig.1) and IL-6 ($r=-0.49$, $p=0.02$) (Fig.2) but no such correlation was found in the placebo group (Table IV).

Table I. The demographic and clinical characteristics of treatment and placebo group

	Rosuvastatin (n=25)	Placebo (n=25)	P value
Age (years)	45 ± 0.92	46 ± 0.95	0.38
Sex (F/M)	21/4	23/2	-
Disease duration (years)	5.90 ± 0.98	5.65 ± 0.74	0.50
BMI (Kg/m^2)	24.29 ± 0.92	24.17 ± 0.82	0.14
Systolic blood pressure (mm Hg)	122 ± 1.90	121 ± 1.68	0.34
Diastolic blood pressure (mm Hg)	82 ± 1.36	81 ± 1.26	0.41
Hb (g/dl)	11.09 ± 0.29	11.06 ± 0.31	0.86
Uric acid (mg/dL)	5.01 ± 0.11	4.99 ± 0.14	0.33
Serum creatinine (mg%)	0.90 ± 0.04	0.89 ± 0.02	0.28

F -Female, M- male, BMI- Body mass index, Hb- Hemoglobin

Table II. Effect of rosuvastatin and placebo after 6 months on clinical variables and inflammatory disease activity measures.

	Rosuvastatin	Group	P value	Placebo	Group	P value
Variables	0 week	6 month		0 week	6 month	
ESR(mm1st hr)	40.30±1.7	24.30±1.7	0.01*	35.55±1.43	33.72±1.13	0.10
CRP (mg/dl)	14.35±3.6	5.35±3.6	0.01*	12.81±0.52	11.01±0.52	0. 12
DAS 28 Score	4.56±0.11	2.65±0.13	**0.01***	4.01±0.11	3.94±0.01	0.15
IL-6 (pg/ml)	21.1±0.10	10.2±0.08	**0.02***	20.10±0.11	19.20±0.13	0.24
IL-1 (pg/ml)	198.2±1.1	188.1±1.3	0.10	199.7±2.1	197.8±3.1	0.34
TNF-α (pg/ml)	6.8±1.1	4.1±0.12	**0.03***	6.4±1.2	5.9±0.12	0.18
EPCs	0.01 ± 0.001	0.04 ± 0.001	**0.01***	0.021 ± 0.001	0.029 ± 0.001	0.32
TC (mg/dl)	216.6±11.3	187.6±9.3	**0.01***	209.6±10.6	200±9.03	0.23
HDL (mg/dl)	40.1±2.3	57.4±0.22	**0.02***	42.4±5.4	43.1±0.12	0.09
LDL(mg/dl)	138.7±6.7	110±0.11	**0.03***	122.3±7.2	121±0.23	0.22
TG (mg/dl)	130.2±2.3	126±0.12	0.08	129.01±1.1	128.88±0.12	0.20
Non-HDL(mg/dl)	172.0 ± 46.3	139.3 ± 30.2	**0.01***	170.0 ± 44.3	165.0 ± 40.3	0.21

DAS 28- Disease activity score of 28 joints, ESR- Erythrocyte sedimentation rate, CRP-C-reactive protein, TC-Total Cholesterol, TG-Triglycerides, HDL-High density lipoprotein LDL-Low density lipoprotein, IL-1- Interleukin-1,IL-6 Interleukin-6, TNF-α -Tumour necrosis factor-α, EPCs-Endothelial Progenitor Cells. *p<0.05, Statistically significant

Table III. Univariate correlation of EPC with clinical disease variables at baseline of RA patients on rosuvastatin and placebo.

	RA		**Placebo**	
	(n=25)		**(n=25)**	
Variables	r	P value	r	P value
Disease Duration	0.10	0.49	0.26	0.63
DAS 28	-0.45	**0.01 ***	-0.49	**0.02***
ESR	0.36	0.19	0.16	0.39
CRP	0.38	0.18	0.40	0.10
IL-6	0.36	0.19	0.39	0.20
IL-1	0.35	0.17	0.49	0.10
TNF-α	-0.48	**0.03***	- 0.41	**0.01***

DAS 28- Disease activity score of 28 joints, ESR- Erythrocyte sedimentation rate, CRP- C-reactive protein, IL-1 -Interleukin-1, IL-6- Interleukin-6, TNF-α -Tumour necrosis factor-α. *p<0.05, Statistically significant

Table IV. Univariate correlation of EPC with clinical disease variables in RA after treatment with rosuvastatin and placebo

	RA		**Placebo**	
	(n=25)		**(n=25)**	
Variables	r	P value	r	P value
Disease Duration	0.30	0.18	0.26	0.43
DAS 28	-0.49	**0.03 ***	0.35	0.12
ESR	0.38	0.17	0.16	0.49
CRP	-0.44	**0.02***	0.19	0.41
IL-6	-0.49	**0.02***	0.12	0.42
IL-1	0.40	0.09	0.19	0.40
TNF-α	-0.42	**0.04***	0.34	0.12

Data are means ± SD. DAS 28- Disease activity score of 28 joints, ESR-Erythrocyte sedimentation rate, CRP-C-reactive protein, IL-1-Interleukin-1, IL-6 -Interleukin-6, TNF-α Tumour necrosis factor-α . *p<0.05, Statistically significant

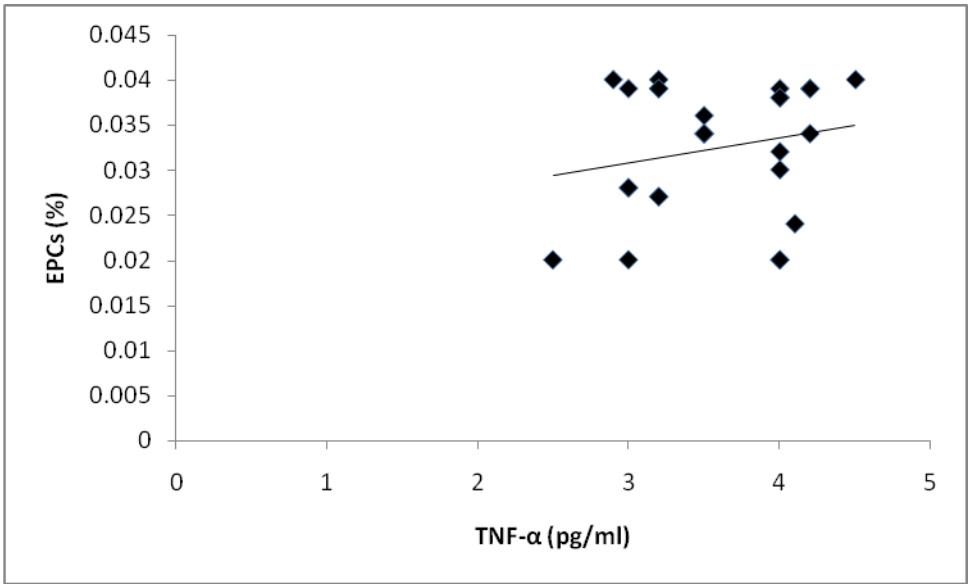

Fig.1 Correlation of Endothelial progenitor cells(EPCs) with Tumour Necrosis Factor-alpha (TNF-α) after treatment with rosuvastatin

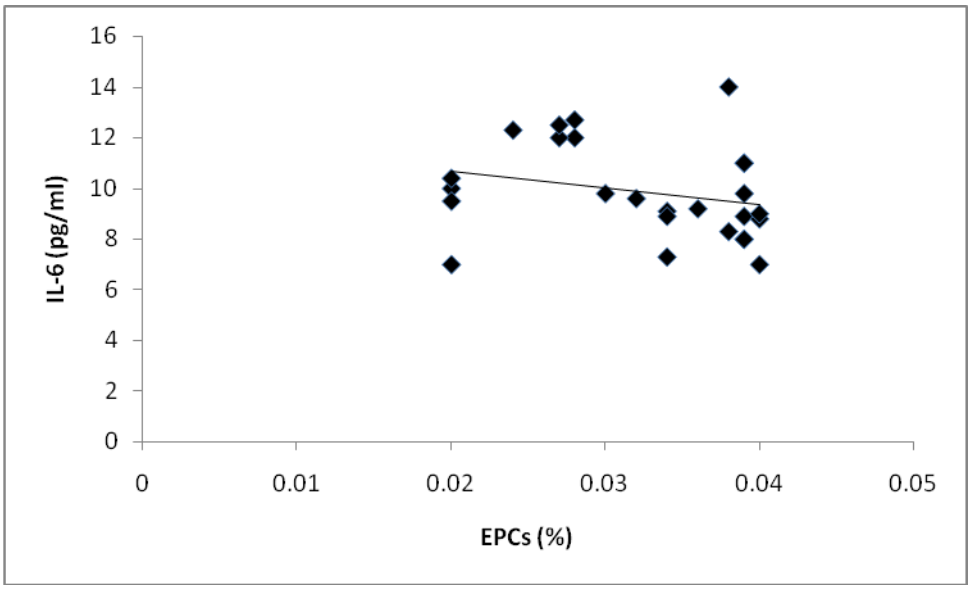

Fig.2 Correlation of Endothelial progenitor cells (EPCs) with Interleukin-6 (IL-6) after treatment with rosuvastatin

Discussion:

This is the first study to demonstrate the therapeutic efficacy of rosuvastatin to improve EPCs population in active RA on conventional anti-rheumatic drugs coupled with reduction in inflammation.

There is an increased prevalence of cardiovascular morbidity and mortality in RA due to premature atherosclerosis [14]. EPCs are a unique population of bone marrow derived haemopoietic stem cells which have reparative potential in overcoming endothelial damage and thus protect against atherosclerotic vascular damage. However, EPC numbers and function are adversely affected in RA and other inflammatory diseases [6-8]. There is homing of EPCs to sites of endothelial damage, including inflamed synovium in RA [15]. EPC mobilization from the bone marrow is impaired due to bone marrow dysfunction. Aspects of EPC proliferation, differentiation and apoptosis are affected by inflammatory mediators like cytokines, CRP, LDL etc. All these contribute to EPC depletion, impaired vasculoprotective effect and increased CV risk in RA. EPCs predict the occurrence of CV events and cardiovascular death [16]. Because of their potential role in vascular repair, the amount of circulating EPCs is

considered a surrogate marker for vascular dysfunction and cardiovascular risk [17] and thus a promising tool in cell therapy for cardiovascular diseases especially in connective tissue diseases. On the other hand, intravenous transfer of competent bone marrow in animals prevents the development of atherosclerotic lesion. Hence an intriguing opportunity has emerged consisting of resurrecting the ability of bone marrow derived vascular progenitors to protect and repair damaged arterial wall [18]. HMG CoA reductase inhibitors have been shown to enhance the proliferation, migration and survival of EPCs in CAD and diabetes [10, 11]. However, the positive effect of this class has not yet been investigated in RA. Against this background, we investigate the effect of rosuvastatin in EPCs in RA patients who did not have any intrinsic CV risk factor or disease.

Our data demonstrates a depletion of peripheral EPCs in patients with active RA. The results of this study add to previous findings showing a reduced number of EPCs in patients with disease associated with an increased CV risk for example, type I and type 2 diabetes. [19, 20]. Moreover EPC depletion in the present study correlates with disease activity, suggesting the pivotal role of inflammation in reducing number of EPCs. This is consistent with the previous study shown an inverse correlation between the disease activity assessed by the DAS28 and CD34+/CD133+/KDR+ cells in the peripheral blood recently reported by Grisar [6]. However, in the presence of endothelial dysfunction, EPC depletion and dysfunction in RA occurs even at low disease activity, though this decrease failed to reach statistical significance. In the present study, treatment with rosuvastatin (10mg/day) for six months improved EPC numbers in RA patients as compared to placebo. This finding is consistent with previous reports that demonstrated increased EPC concentration after statin treatment in CAD and diabetes [10, 11].

In patients with active arthritis, it is not known whether enhancing the level of EPCs will simultaneously augment the arthritic process, as EPCs contribute to hypervascularization of synovium. In the present study, treatment of RA with rosuvastatin for six months did not aggravate the arthritis. On the continuity, improvement in EPC population was accompanied by an improvement in inflammatory disease activity. The possible mechanism for improving EPCs in our study by rosuvastatin may involve the activation of phosphatidylnositol 3- kinase (PI3K) and protein kinase B (Akt) pathway, as this pathway regulate the cell cycle progression in EPCs. Recent evidence indicates that atorvastatin could reduce the senescence of EPCs by increasing the cell cycle-inhibitory protein [21].

Elevated levels of CRP and IL-6 correlates with accelerated atherosclerosis in RA patients [22] and it has been demonstrated that CRP has the potential to decrease the angiogenic functon of EPCs [23, 24]. In our study, level of CRP was elevated in both groups which is associated with increased level of TNF-α and IL-6 coupled with reduced number of EPCs. After treatment with rosuvastatin, there was significant decrease in CRP, DAS 28, ESR and cytokine levels i.e. TNF-α and IL-6 which may explain its beneficial effect on EPCs. Our findings are consistent agreement with other studies who predicted a significant decrease in CRP levels after statin treatment in different patient populations. Tikiz et al, has demonstrated a reduction in CRP levels and improved the clinical status in RA after treatment with simvastatin (20mg/day for 8 weeks) [25]. Additionally, in a recent study, McCarey, et al investigated the effects of atorvastatin in patients with RA and found significantly improved clinical status measured by DAS 28 and decreased CRP and IL-6 levels at the end of 6 months [26]. Our findings are consistent with these studies. From this point of view, rosuvastatin seems to be an important drug in RA patients having high CV risk.

In the present study levels of pro-inflammatory cytokines including TNF-α, IL-6 and IL-1 were elevated in both the study groups coupled with EPC depletion. In a recent study, it has been indicated that TNF-α and IL-6 play a pivotal role in reducing EPC levels in RA patients indicating that microinflammtory state is related to the number of EPCs [27]. However, present study has also found significant correlation between EPC and TNF-α suggesting TNF-α has a potential to decrease the number of EPCs in RA. In a recent study, simvastatin was found to decrease TNF-α levels in RA patients [25]. In our study, a significant correlation was found between EPCs and TNF-α and IL-6 after treatment with rosuvastatin as compared to placebo and our results add to the previous findings. This beneficial effect was may be attributed due to antiinflammatory and immunomodulatory properties of the rosuvastatin, which was independent of their cholesterol-lowering function. Recent data suggests that statin has the ability to inhibit the Nuclear factor kappa B (NF-kB) which regulates the expression of cytokines and has central role in inflammatory rheumatic diseases [28]. On the basis of these findings, it is also tempting to speculate that the rosuvastatin-induced downregulation of cytokine production in RA may involve, in part, a loss of functional nuclear factor-kB.

The lipid profile in RA has often been described as ‘‘pro-atherogenic’’, based on decreased HDL cholesterol (HDL-C) and increased LDL:HDL-C ratio in both active and treated RA which is responsible for CV events [29]. The serum TC and HDL-C levels in RA are inversely correlated with disease activity, suggesting a potential role for inflammation in the atherogenic profile and the higher atherosclerotic risk observed in RA [30]. In our study, we also observed altered lipid profile characterized by an increased levels of serum Total Cholesterol, LDL- Cholesterol , non-HDL Cholesterol and triglyceride levels and a decrease in HDL-C levels that are consistent with the previously reported studies. In addition, in hypercholesterolemic patients, EPC function was impaired and

EPC numbers were reduced and inversely correlated with LDL plasma levels [31]. Statins have previously been shown to increase EPC levels in patients with coronary artery disease and in patients with chronic heart failure [10, 32]. A previous study showed that lipid-lowering therapy with 10 mg simvastatin enhanced EPC levels in chronic heart failure patients, also consistent with a pleiotropic cholesterol-independent effect of statins on EPC levels [33]. Previous studies of rosuvastatin has shown significant improvement in HDL-C and non-HDL in hypercholesteremic patients [34]. In the present study, 24 weeks of treatment with rosuvastatin 10 mg followed by important changes in lipid spectrum that were similar to those previously reported: increase in HDL-C levels, decrease in total cholesterol, LDL and non-HDL levels which are equally effective in enhancing EPC levels. This is the first study which shows the effect of rosuvastatin on non-HDL in RA patients This suggests a more important role for LDL-reduction and increase in HDL in contrast to potential pleiotropic effects of statin therapy for the observed EPCs increase in our patients.

In conclusion, this is the first study to show that rosuvastatin improves the inflammatory disease activity and increases the number of EPCs in patients with RA. Its beneficial effect may be attributed to its lowering levels of CRP and TNF-α and IL-6. In present study, potential dual effects in reducing inflammation and improving EPCs may accrue with the use of rosuvastatin in RA. These results offer a novel view point on the anti-inflammatory effect of statin on EPCs in RA. This would serve as a novel therapeutic target for preventing CV risk associated with reduced number of EPCs in RA as the effect of rosuvastatin on this population have not been previously studied.

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Conflict of Interest None

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