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# Receptor for advanced glycation end products (RAGE) and its inflammatory ligand EN-RAGE in non-diabetic subjects with pre-mature coronary artery disease

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ABSTRACT

*Objective:* Inflammation participates in atherosclerosis from its inception onwards. RAGE (receptor for advanced glycation end products) and its natural pro-inflammatory ligand, EN-RAGE (extracellular newly identified RAGE-binding protein) have been implicated in various inflammatory diseases. In present study, we determined the expression of RAGE and EN-RAGE in peripheral blood mononuclear cells (PBMCs) of subjects with pre-mature coronary artery disease (CAD) for the first time.
*Methods and results:* The study patients were angiographically proven non-diabetic patients with pre-mature CAD (Group I; $N = 100$) and control group comprised of subjects with coronary risk factors and without coronary artery lesions (Group II; $N = 40$). Semi-quantitative RT-PCR was performed to determine transcriptional expression of RAGE and EN-RAGE in PBMCs. Soluble RAGE (sRAGE) and C-reactive protein (hsCRP) levels were determined in serum of all study subjects using immunoassays. A significantly increased transcriptional expression of RAGE and EN-RAGE in PBMCs ($p < 0.01$) of Group I patients was observed. Increased circulating hsCRP ($p < 0.01$) levels and decreased sRAGE ($p < 0.01$) levels were observed in Group I as compared with the Group II subjects. Severity of disease determined by Gensini score was found to be positively correlated with transcriptional expression of RAGE ($r = 0.530$) and EN-RAGE ($r = 0.323$). EN-RAGE expression revealed a strong association with RAGE ($r = 0.326$), hsCRP ($r = 0.251$) and a negative association with sRAGE ($r = -0.222$).
*Conclusions:* Increased expression of RAGE and EN-RAGE in non-diabetic pre-mature CAD and various associations discussed may amplify several cellular perturbations and thus significantly contribute to the pathophysiology of CAD.



## 1. Introduction

Worldwide, cardiovascular disease (CVD) is a major cause of mortality and accounts for 80% of early deaths in developing countries. The increasing incidence of coronary artery disease (CAD) in young Indian population (<55 years) is attributed to global industrialization, stressed life, lack of exercise, smoking and alcohol consumption as well as to other nutritional and lifestyle factors [1,2].

RAGE (receptor for advanced glycation end products) is a member of the immunoglobulin superfamily. It is expressed on the surface of various cells as endothelium, mononuclear phagocytes, lymphocytes and smooth muscle cells. The inflammatory role of RAGE and EN-RAGE (extracellular newly identified RAGE-binding protein) interaction is well established in various chronic inflammatory disorders [3–6]. C-truncated RAGE (endogenous secretory RAGE, esRAGE) isoforms lacking the transmembrane domain and other RAGE isoforms, cleaved proteolytically from the membranous receptor via MMPs, circulate in the plasma, where they can act as a decoy for RAGE ligands. These secreted variants together, represent the total amount of soluble RAGE (sRAGE) that can be detected in the blood stream [7,8]. Recent studies have also reported that reduced levels of sRAGE serve as an important biomarker in hypertension [9], Alzheimer disease, vascular dementia [10], mild cognitive impairment [11] and CAD [12]. Association of decreased levels of esRAGE with type 1 diabetes [13], metabolic syndrome [14] and arterial stiffness [15] has also been suggested.

EN-RAGE, an inflammatory ligand of RAGE, is a member of the S100 protein family, also referred as S100A12. EN-RAGE acts as a potent chemoattractant; its ligation with RAGE on the endothelium, mononuclear phagocytes and lymphocytes triggers cellular activation with the generation of the key pro-inflammatory mediators as interleukin (IL)-1β and tumour necrosis factor (TNF)-α [16].

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Therefore, binding of RAGE with EN-RAGE, may further promote underlying inflammation in atherosclerosis and its related manifestations such as CAD.

In the present study, we attempted to determine the expression of RAGE and EN-RAGE in peripheral blood mononuclear cells (PBMCs) and also their correlation with the severity of disease and circulating levels of sRAGE and hsCRP in the non-diabetic subjects with pre-mature CAD.

## 2. Materials and methods

### 2.1. Study population

A total of 140 non-diabetic subjects visiting Cardiology Clinic at Nehru Hospital for coronary angiography owing to CAD (Group I; $N = 100$) or because of a clinical suspicion of CAD in subjects with multiple coronary risk factors (Group II; $N = 40$) were enrolled for the study. Subjects with negative treadmill test (TMT) or positive TMT but having no lesion on angiography served as controls. Exclusion criteria included myocardial infarction 2 months before the study, evidence of hemodynamically significant valvular heart diseases, congenital heart disease, surgery or trauma 1 month before the study, known cardiomyopathy, known malignant diseases, febrile conditions, acute or chronic inflammatory disease, overt congestive heart failure, renal insufficiency and abnormal liver function. Individuals having a concentration of hsCRP $\geq$10 mg/l, a level considered to be indicative of clinically relevant inflammatory conditions were also excluded [17]. Blood pressure measurements were taken as per JNC VI criteria at the time of sampling [18].

Pre-mature CAD is defined as CAD occurring before age 65 years in women and 55 years in men [19]. The mean age for first presentation of acute myocardial infarction in Indians is 53 years irrespective of sex [20]. Therefore, in the present study we recruited subjects of either sex with aged $\leq$55 years. Data on demographic factors, body mass index [BMI (kg/m$^2$)], smoking habits, alcohol consumption, diet and family history of cardiovascular disease in first-degree relatives was recorded. All study participants underwent a standard clinical examination. Alcohol intake and cigarette/beedi (a local type of tobacco) smoking was dichotomized into ever vs. never, with ever smoking defined as having smoked daily for a year or more. Many patients had quit alcohol intake/smoking after onset of their CAD, hence, designated as ever rather than current or former. Hypertension was defined as resting systolic blood pressure >140 mmHg and diastolic blood pressure >90 mmHg or use of any anti-hypertensive agent. The Group I subjects were receiving identical drug therapy including ACE inhibitors, nitrates, heparin, β-blockers or statins.

The study was performed according to the ethical standards detailed in the Declaration of Helsinki [21]. The study was approved by the Institutional Ethics Committee. Before participation in the study, an informed written consent was obtained from each subject after explaining the protocol.

### 2.2. Sampling

Venous blood was collected in the morning from the overnight fasting subjects from anticubital vein into plain sterile tube for serum and in EDTA for plasma just before the angiography and/or after TMT (in subjects who had negative TMT). Serum/plasma was separated and stored at −80 °C for further analysis.

### 2.3. Severity of coronary artery disease

The severity of coronary atherosclerosis in patients was assessed using the Gensini score [22], which grades narrowing of the lumens of the coronary arteries. The score was given as 1 for 1–25% narrowing, 2 for 26–50% narrowing, 4 for 51–75% narrowing, 8 for 76–90% narrowing, 16 for 91–99% narrowing and 32 for total occlusion. The score is then multiplied by a factor that takes into account the importance of the lesion's position in the coronary arterial tree, for example, 5 for the left main coronary artery, 2.5 for the proximal left anterior descending coronary artery (LAD) or proximal left circumflex coronary artery (LCX), 1.5 for the mid-region of the LAD, and 1 for the distal LAD or mid-distal region of the LCX. Gensini score was expressed as the sum of the scores for all the coronary arteries.

### 2.4. Measurement of lipid and lipoprotein profile

Serum total cholesterol (TC), triglycerides (TG) and high-density lipoprotein cholesterol levels (HDL-C) levels were measured with standard enzymatic kits (Accurex Biomedical Pvt. Ltd.). Low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein (VLDL-C) values were calculated using Friedwald's formula [23].

### 2.5. Determination of circulating levels of sRAGE and hsCRP

Circulating levels of sRAGE (DRG00, R&D Systems, USA) and hsCRP (RK010A, Hyphen Co., France) were determined in all the study subjects using commercially available enzyme-linked immunoassays as per manufacturer's instructions. The immunoassay used for determination of soluble RAGE measures the total pool of soluble RAGE which is generated either by splicing or cleavage (e.g. sRAGE or esRAGE).

### 2.6. Transcriptional expression (mRNA) of RAGE and EN-RAGE

PBMCs were isolated using Ficoll gradient method as described by Boyum [24]. Semi-quantitative RT-PCR was performed for determining the transcriptional expression (mRNA) of RAGE and EN-RAGE [25] in PBMCs by using human specific primer pairs (Table 1).

### 2.7. Statistical analysis

Statistical analysis was performed using SPSS 14.0 for Windows (SPSS Inc.). The Kolmogorov–Smirnov test of normality was used to verify whether the distribution of variables followed a Gaussian pattern. Data was presented as mean ± S.D. or percentages. Mean levels of different groups were compared by *t*-test. Correlations between variables were analyzed with Pearson's coefficient. Logistic regression analysis was used to determine the association between the severity of the CAD and all other variables considered in the present study.

## 3. Results

### 3.1. Baseline clinical and laboratory characteristics

The clinical and laboratory features of the subjects are shown in Table 2. The two study groups were comparable with respect to

**Table 1**
Characteristics of primers used for RT-PCR.

| Gene | Primer | Sequence | Fragment length | PCR cycles |
|---|---|---|---|---|
| RAGE | Forward | 5′-CACACTGCAGTCGGAGCTAA-3′ | 192 bp | 40 |
| | Reverse | 5′-GCTACTGCTCCACCTTCTGG-3′ | | |
| EN-RAGE | Forward | 5′-ATGACAAAACTTGAAGAG-3′ | 280 bp | 35 |
| | Reverse | 5′-CTACTCTTTGTGGGTGTG-3′ | | |
| β-actin | Forward | 5′-GAATTGCTATGTGTCTGGGT-3′ | 257 bp | 27 |
| | Reverse | 5′-CATCTTCAAACCTCCATGATG-3′ | | |

**Table 2**
Baseline characteristics of all the study subjects.

| Variables | Group I ($N=100$) | Group II ($N=40$) | Level of significance |
|---|---|---|---|
| *Age (years)* | | | |
| Range | 24–55 | 24–55 | NS |
| Mean ± S.D. | 44.4 ± 7.9 | 41.6 ± 7.5 | NS |
| Sex ratio (M:F) | 81:19 | 27:13 | NS |
| BMI (kg/m$^2$) | 22.68 ± 1.95 | 22.55 ± 2.95 | NS |
| *Blood pressure* | | | |
| SBP (mmHg) | 125.47 ± 10.93 | 121.95 ± 9.21 | NS |
| DBP (mmHg) | 81.65 ± 9.19 | 77.50 ± 7.42 | **0.012**$^{*}$ |
| *Lipid profile* | | | |
| TC (mg%) | 190.01 ± 35.99 | 179.45 ± 29.65 | NS |
| TG (mg%) | 153.71 ± 56.60 | 132.71 ± 39.29 | **0.034**$^{*}$ |
| HDL (mg%) | 42.87 ± 9.14 | 44.92 ± 8.4 | NS |
| LDL (mg%) | 116.40 ± 32.57 | 107.89 ± 28.77 | NS |
| *Soluble markers* | | | |
| hsCRP (μg/ml) | 5.76 ± 3.12 | 1.67 ± 1.90 | **<0.0001**$^{***}$ |
| sRAGE (pg/ml) | 892.39 ± 508.68 | 1611.90 ± 677.31 | **<0.0001**$^{***}$ |
| *Risk factors* | | | |
| Hypertension (%) | 31 | 0 | – |
| Smoking (%) | 47 | 38 | NS |
| Alcohol intake (%) | 28 | 35 | NS |
| Veg (%)/non-veg (%) | 55/45 | 38/62 | NS |
| Family history of CVD (%) | 50 | 10 | **0.013**$^{*}$ |
| *Medications* | | | |
| Aspirin | 63 | 0 | – |
| Nitrate | 32 | 0 | – |
| Calcium channel blockers | 44 | 0 | – |
| ACE inhibitors | 46 | 0 | – |
| Angiotensin receptor blockers | 14 | 0 | – |
| Beta blockers | 39 | 0 | – |
| Statins | 71 | 0 | – |

Data mean ± S.D. or percentage.
$^{*}$ $p<0.05$.
$^{***}$ $p<0.001$.

age, BMI, smoking, alcohol intake, etc. and levels of TC and HDL-C ($p>0.05$), whereas serum TG levels were found to be significantly higher in Group I subjects ($p<0.05$). We observed no significant difference in the mean systolic blood pressure (SBP) recordings ($p>0.05$) in the two groups, whereas, diastolic blood pressure (DBP) recordings were found to be significantly higher in Group I subjects ($p<0.05$).

**Table 3**
Associations between soluble markers and severity of coronary artery disease as determined by Pearson's correlation analysis.

| Variable | GS | RAGE | EN-RAGE | sRAGE | hsCRP |
|---|---|---|---|---|---|
| GS | – | 0.530$^{**}$ | 0.323$^{**}$ | −0.342$^{**}$ | 0.547$^{**}$ |
| RAGE | – | – | 0.326$^{**}$ | −0.059 | 0.157 |
| EN-RAGE | – | – | – | −0.222$^{*}$ | 0.251$^{*}$ |
| sRAGE | – | – | – | – | −0.326$^{**}$ |
| hsCRP | – | – | – | – | – |

$^{*}$ $p<0.05$.
$^{**}$ $p<0.01$.

### 3.2. Circulating levels of sRAGE and hsCRP

The levels of sRAGE were determined in CAD patients and control subjects. sRAGE levels were observed to be significantly lower in serum of patients with CAD (892.39 ± 508.68 pg/ml) as compared to the controls (1611.90 ± 677.31 pg/ml) ($p<0.0001$).

We observed higher hsCRP levels in serum of Group I subjects as compared with the Group II (5.76 ± 3.12 vs. 1.68 ± 1.9 μg/ml; $p<0.0001$).

### 3.3. Transcriptional (mRNA) expression of RAGE and EN-RAGE

The transcriptional expression of genes coding for RAGE and EN-RAGE within blood mononuclear cells derived from Group I ($N=100$) subjects was significantly higher ($p<0.01$) than that observed in blood mononuclear cells from subjects of Group II ($N=40$) (Fig. 1).

### 3.4. Statistical analysis

Correlational analysis was performed to survey the relationship among laboratory measures of variables from cohorts of CAD patients with severity of the disease. The results are presented in Table 3.

The sensitivity and specificity were calculated for each possible threshold value of estimated probability for the respective group.

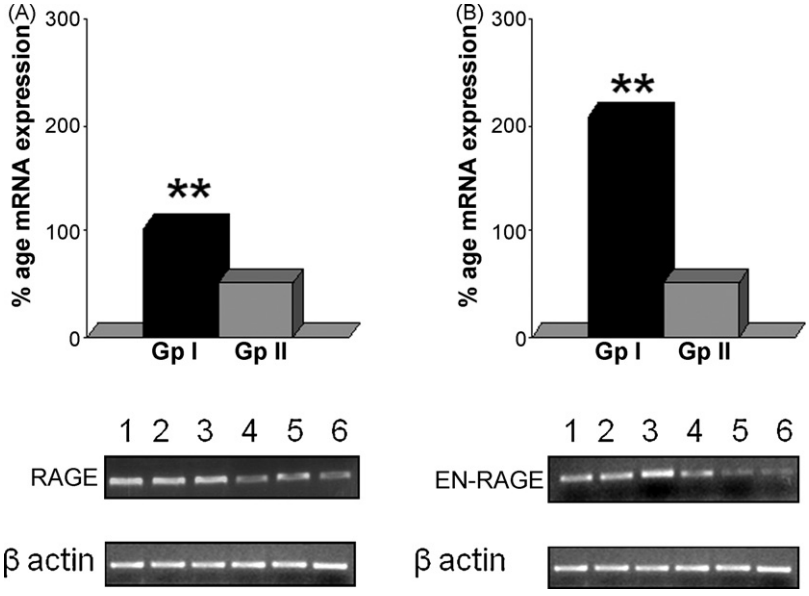


**Fig. 1.** Depicts the mRNA expression of RAGE (panel A) and EN-RAGE (B) in PBMCs isolated form subjects of pre-mature coronary artery disease (Gp I; lanes 1–3) and subjects of risk control group (Gp II; lanes 4–6) as determined by semi-quantitative RT-PCR. $^{**}p<0.01$.

With EN-RAGE, an optimal sensitivity of 93% and specificity of 87% was achieved, whereas, hsCRP demonstrated 87% sensitivity and 70% specificity. sRAGE revealed a sensitivity of 72.5% and specificity of 77% for prediction of CAD in these subjects with pre-mature coronary artery disease. Further, logistic regression analysis revealed that sRAGE, hsCRP and EN-RAGE statistically were a potential predictors of disease. These three variables cumulatively demonstrated a high sensitivity (87.5%) and specificity (95%).

## 4. Discussion

This is the first study of its kind where we demonstrate that mRNA levels of RAGE and its ligand EN-RAGE are up-regulated in non-diabetic patients with pre-mature CAD. In addition, significantly augmented levels of hsCRP and decreased levels of sRAGE were observed in the serum of patients with CAD as compared with controls. Further, this study demonstrates association of RAGE and EN-RAGE with sRAGE, hsCRP and severity of CAD in these patients for the first time.

Our data supports the findings of Haidari et al. [26] and Tataru et al. [27] who independently reported increased hsCRP levels in subjects with stable CAD and also in subjects with acute coronary syndrome (ACS). Further, they observed a positive correlation of hsCRP with severity of disease. These findings are further strengthened by our observations and highlight the importance of hsCRP as a marker of disease activity. On the contrary, studies by Rifai et al. [28] and Abdelmouttaleb et al. [29] observed increased hsCRP levels in similar set of subjects but there was no significant correlation of hsCRP with the severity of CAD. In the present study, we did not observe a significant correlation of hsCRP levels with any of the other classical risk factors like age, BMI, lipids and lipoproteins in Group I subjects. Our results are in contrast to the findings of Mendall et al. [30] and Tracy et al. [31] who reported a positive association of hsCRP with age and BMI in subjects with coronary heart disease. These discrepancies may be attributed to the differences in population characteristics and the sample size in both these studies.

sRAGE has been documented as an anti-atherogenic molecule and low levels of sRAGE were reported to be significantly associated as a risk factor for CAD in a non-diabetic Italian male population [12]. In the present study, a significant and independent association of decreased levels of sRAGE with the presence of CAD, increased levels of hsCRP, increased transcriptional expression of EN-RAGE in PBMCs, further strengthen the anti-atherogenic nature of sRAGE. We also observed that CAD subjects who were hypertensive had low levels of sRAGE as compared to the CAD subjects who were normotensive. These observations are consistent with earlier observations of Geroldi et al. [9], that levels of sRAGE were decreased in patients with hypertension and increased the possibility that sRAGE may play a role in arterial stiffness and its related complications. Our data demonstrated absence of any significant correlation of sRAGE with BMI and similar findings were reported by Geroldi et al. [9]. However, Koyama et al. [14] in their study reported a significant inverse correlation of esRAGE and BMI in subjects with metabolic syndrome. All other studies with sRAGE till date have been conducted in either Caucasian or Asian (Japanese) population and this is the first ever study to be conducted in Indian patients.

Recently, Santilli et al. [32] reported significantly lower levels of sRAGE in hypercholesterolemic subjects as compared to normocholesterolemics. The subject population in our study mainly included normocholesterolemic CAD subjects and only 9% of CAD subjects had high cholesterol levels. Also, analysis on the basis of cholesterol levels in our study revealed no statistically significant difference in any of the variables under consideration (data not shown). They also reported higher sRAGE levels in patients with previous myocardial infarction who were on statin intake. As far as atorvastatin intake is concerned, its influence on sRAGE levels cannot be excluded on the basis of stratification of subjects in Group I in the present study. In the present study, we observed a lack of correlation between sRAGE and RAGE mRNA expression in study subjects which could be partially attributed to the drug intake by these subjects. Categorization of the subjects on basis of atorvastatin intake did not reveal any significant difference in any variable except hsCRP (data not shown). The possible reasons for a lack of correlation between sRAGE and RAGE levels, could be (i) no follow-up of the subjects receiving atorvastatin treatment; (ii) categorization of the study subjects on the basis of duration of atorvastatin intake irrespective of their dosage regimen. Further studies in larger number of subjects are required to determine the effect of atorvastatin intake on these variables.

In an isolated report, Burke et al. [33] demonstrated increased expression of RAGE and EN-RAGE in the coronary atherosclerotic plaques obtained from diabetic CAD cadavers. In addition, these authors related increased expression of RAGE and EN-RAGE with necrotic core expansion, thinning of the fibrous cap and plaque instability. An augmented expression of RAGE and EN-RAGE in the present study, along with a positive correlation of these genes with the severity of disease, further corroborates the concept that RAGE-EN-RAGE axis participates as one of the mechanisms, that may assist in explaining its role in various diseases characterised by underlying inflammation, recruitment of leukocytes, apoptosis, and macrophage cell death [33–36].

The ligation of EN-RAGE with cellular RAGE may also activate the downstream signalling pathway such as NF-κB, in addition to the increased expression of adhesion molecules and cytokine release from the lymphocytes [3,37]. Thus, finding a positive correlation among serum hsCRP levels, mRNA expression of RAGE and EN-RAGE may represent an attractive model to explain how these molecules contribute to the pathophysiology of inflammatory diseases like atherosclerosis. Furthermore, logistic regression analysis revealed sRAGE, hsCRP and EN-RAGE as potential predictors of CAD with high sensitivity and specificity and thus could serve as potential biomarkers.

Gene functions of spliced forms may influence the regulatory effects of the constitutive human genes. As for RAGE, the production of endogenous secretory RAGE by alternative splicing, may influence the actions of ligands on the full form of RAGE, located on the plasma membrane [38]. In the current study, along with increased RAGE mRNA levels, one would expect a similar increase in sRAGE levels. It may suffice to say that RAGE mRNA levels comprise of full length RAGE and soluble isoforms correspond to a small fraction and was low in patients. The likely mechanisms could be that the increase in RAGE mRNA levels were mainly in the full length form and due to changes occurring in the ratio of RAGE splice variants. Other possibility might be that comparatively lesser sRAGE in patients with increased RAGE expression which is suggestive of some epigenetic modifications or post-translational mechanisms that could be responsible for attenuated splicing in patients as compared to the controls. At the same time one could not ignore the recently proposed mechanisms for post-translational modifications of full length RAGE like shedding of ectodomain and proteolysis by MMP-9 or ADMA10 [39,40]. However, we observed low levels of sRAGE in CAD subjects, which could be explained as shedding may be modulated by binding of pro-inflammatory ligands (e.g. EN-RAGE) or alternatively, low levels of sRAGE may not be sufficient as a decoy for pro-inflammatory ligands and therefore, do not protect against inflammation. Another important point to be considered is that although we have studied PBMCs as cellular model for gene expression studies, RAGE is expressed by a variety of other cells such as endothelial cells and smooth muscle cells, the cell types relevant in CAD. The relative abundance of different isoforms of RAGE may vary accordingly among different cells

and tissues and among individuals and/or conditions and could be another explanation for our observations. Our data is supported by Hudson et al. [41], who reported the prevalence of RAGE in aortic smooth muscle cells (AoSMCs) and lungs and observed that full length isoform of RAGE accounts for 70 and 80% of the detected transcripts respectively. Such diversity could be a precipitating factor which endows subjects with presence/absence of risk factors to the development of various complications [42]. Further, studies are needed to clarify the exact significance of the co-expression of full length type RAGE and antagonistic RAGE variants in various diseased pathophysiologies and to future preventive strategies.

Up-regulation of inflammatory NF-κB, COX-2/mPGES-1 (microsomal Prostaglandin E2 synthase) and matrix metalloproteinases (MMPs) and augmented RAGE expression have been demonstrated in carotid arteries of diabetic subjects [43]. Enhanced MMPs synthesis as a consequence of RAGE over-expression may possibly represent a crucial step in the pathophysiology of plaque instability. In support, we observed augmented expression of MMP-9 in these subjects and along with transcriptional expression of RAGE and EN-RAGE (Unpublished data). Finding augmented expression of RAGE and EN-RAGE in PBMCs of these subjects' further points towards the presence of heightened intracellular oxidative stress.

Increased RAGE and EN-RAGE expression in normo-glycemic conditions could be partially explained by findings of Cuccurullo et al. [44]. These authors demonstrated modulation of RAGE expression in human plaques in a glucose-independent manner by inhibition of macrophage myeloperoxidase and therefore, suggested pro-atherogenic role of RAGE in various clinical settings characterised by underlying inflammation and high infiltration of activated macrophages. The data obtained from the present study, highlights the fact that RAGE and EN-RAGE axis co-operates with pro-inflammatory cytokines in an autocrine, paracrine and endocrine manner at the site of atherosclerotic lesion and contributes to the disease progression. Blockade of RAGE and EN-RAGE interaction in early stages of the inflammatory process may inhibit inflammation and prevent further sequels of atherosclerosis. So far, the source of immunological agonists for PBMCs has not been fully elucidated in such diseases where these cells are attributed with increased production of various inflammatory cytokines.

The limitations of this study are the relatively smaller number of participants and the limitations inherent to any cross-sectional design, specifically that a single sample at a certain point in time may fail to reflect the natural course of the process being studied. Secondly, the ELISA detection system used in the present study could determine total sRAGE levels and could not discriminate between specific soluble RAGE variants (sRAGE and esRAGE). The presence of different co-morbid conditions in a CAD population using multiple drugs represents an additional limitation in the interpretation of the data.

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