Increased Levels of Endotoxin Core Antibodies and Decreased Levels of sCD 14 Indicate Chronic Endotoxemia in Coronary Artery Disease (CURES-127)

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Abstract

Introduction: Endotoxin is hypothesized to play an important role in chronic inflammation associated with coronary artery disease (CAD). The activity of the endotoxin is determined by the relative levels of endotoxin core antibodies (EndoCab), LPS binding protein (LBP) and soluble CD14 (sCD14). Towards this end, we estimated EndoCab, LBP and sCD14 levels in subjects with CAD and correlated them with clinical parameters, serum hormones and cytokine levels.

Methods: The study subjects were recruited from Chennai Urban Rural Epidemiology Study (CURES). Age and gender matched subjects with (n=46) and without (n=45) CAD were selected from Phase III of the study. The levels of serum lipopolysaccharide (LPS), EndoCab and its translocation markers (sCD14 and LBP) along with insulin, glucagon, adiponectin and leptin and pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β were measured by immuno-assays.

Results: Quartile analysis showed significantly higher percentage of CAD subjects in the highest quartile of EndoCab (>26.6 EMU/ml) (CAD-24% Vs Controls 0%) and LPS (>65.05 EU/ml) (CAD-21% vs. Controls 4%), while a reverse trend was seen for sCD14 (>2198398.7 ng/ml) (CAD-1% vs. Control-24%) (p<0.01). LBP levels were not statistically different between the groups. While EndoCab levels showed positive association with LPS, LBP, sCD14, hsCRP, HOMA-IR and glucagon, sCD14 showed a negative association with most of these markers. Significant increase in the levels of pro inflammatory cytokines TNFα, IL-6 and IL-1β, was also observed in the CAD group when compared to the control.

Conclusions: Significantly elevated levels of EndoCab and decreased levels of sCD14 indicate chronic endotoxin exposure in CAD subjects.

Keywords: CAD; LPS; LBP; sCD14; EndoCAb; Glucagon; Adiponectin; Leptin

Abbreviations: EndoCab-Endotoxin Core Antibody; LPS-Lipopolysaccharide; LBP-LPS Binding Protein; sCD14-souble Cluster Differentiation 14; hsCRP-High Sensitivity C Reactive Protein; IMT-Intima-Media Thickness; HOMA-IR-Homeostatic Model Assessment-Insulin Resistance

Introduction

CAD is increasingly being recognized as a chronic inflammatory disease [1]. A high-fat meal induces low-grade endotoxemia [2] and the levels of endotoxin have been associated with a threefold risk of atherosclerosis [3]. But more than the actual endotoxin levels, the levels of endogenous anti-endotoxin antibodies (EndoCab), LPS binding protein (LBP) and soluble CD14 (sCD14) were found to be more important in determining the activity of endotoxin [4,5]. These three components play an important role in conditions of acute inflammation like septicemia as in post operative surgery [4]. A consistent drop in post-operative levels of circulating EndoCab levels, compared to the pre-operative value following surgery has been reported, due to the consumption of these antibodies in neutralizing endotoxins [6]. CD14 is a glycosylphosphotidylinositol- anchored membrane glycoprotein constitutively expressed on monocytes/macrophages and neutrophils [7]. Cell surface CD14 binds to lipopolysaccharide (LPS) in the presence of LBP resulting in the activation of TLR4 signaling and secretion of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) [8,9]. CD14 also exists as a soluble form (sCD14). sCD14 may be derived by enzymatic

cleavage of mCD14 or might be secreted by liver. Like other acute phase proteins, sCD14 levels increase in the setting of acute inflammatory conditions. Recently, components of microbial translocation have been shown to play an important role in the susceptibility of various chronic diseases [10].

Previous studies have documented systemic low grade endotoxemia in CAD subjects [11]. However, reports on levels of EndoCab, LBP and sCD14 in CAD subjects are scant. Thus, in the present study we measured the levels of LPS, EndoCab, LBP and sCD14 and correlated it with clinical risk factors for CAD (Insulin Resistance (IR), C-Rreactive Protein (CRP), Intimal Medial Thickness (IMT)), pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) and hormones which affect metabolic homeostasis and vasculature (glycogen, adiponectin and leptin).

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Materials and Methods

Study subjects

Study subjects were recruited from the Chennai Urban Rural Epidemiology Study (CURES), an ongoing large epidemiological study conducted on a representative population of Chennai (formerly Madras), the fourth largest city in India. The methodology of the study has been published [12] and has also been depicted in Supplementary Figure 1. Briefly, in Phase-1 of the urban component of CURES, 26,001 individuals were recruited based on a systematic random sampling technique. Fasting capillary blood glucose was determined using the OneTouch Basic glucometer (Lifescan, Johnson & Johnson, Milpitas, CA) in all subjects. In Phase-2, detailed studies of diabetic complications, including nephropathy, neuropathy, retinopathy and cardiomyopathy were performed, and in Phase-3, every 10th individual in Phase 1 was invited to participate in more detailed studies.

Inclusion and exclusion criteria

The inclusion criteria were patients within the normal range of white blood cell to minimize the confounding effect of infection. The exclusion criteria were patients with type-1 diabetes and patients with a previous diagnosis of urolithiasis, liver cirrhosis, congestive heart failure, chronic lung diseases, chronic infections, viral hepatitis, or any known renal diseases. Institutional ethical committee approval from the Madras Diabetes Research Foundation Ethics Committee was obtained (Ref No-MDRF-EC/SOC/2009//05) and written informed consent was obtained from all the study participants. For the present study, 46 subjects with CAD who fall under the inclusion-exclusion criteria and 45 age and gender matched controls were selected from Phase-3 of CURES. CAD was diagnosed based on positive medical history (documented myocardial infarction (MI), angina pectoris and coronary artery bypass graft) and/or ischemic changes on a conventional 12-lead ECG which included ST-segment depression (Minnesota codes 1-1-1 to 1-1-7) or Q-wave changes (Minnesota codes 4-1 to 4-2) [13]. Absence of CAD was based on absence of history of angina or myocardial infarction and normal ECG.

Anthropometric and biochemical parameters

Anthropometric measurements including height, weight, and waist circumference, were obtained using standardized techniques. The body mass index (BMI) was calculated as the weight in kilograms divided by the square of height in meters. Fasting plasma glucose (FPG) (glucose oxidase-peroxidase method), serum cholesterol (cholesterol oxidase-peroxidase-amidopyrine method), serum triglycerides (glycerol phosphate oxidase-peroxidase-amidopyrine method), high density lipoprotein cholesterol (HDL-C) (direct method-polyethylene glycol-pretreated enzymes), and creatinine (Jaffe's method) were measured using a Hitachi-912 Autoanalyser (Hitachi, Mannheim, Germany). The intra- and inter assay coefficient of variation for the biochemical assays ranged between 3.1% and 5.6%. Glycated hemoglobin (HbA1c) was estimated by high pressure liquid chromatography using a variant machine (Bio-Rad, Hercules, CA). The intra- and inter-assay coefficient of variation of HbA1c was less than 5%. The plasma concentrations of high-sensitivity C-reactive protein (hsCRP) were measured by a highly sensitive nephelometric assay. The intra- and the inter-assay coefficients of variation for hsCRP were 4.2% and 6.8%, respectively, and the detection limit was 0.15 mg/L.

Detection of serum LPS and translocation markers

Serum lipopolysaccaride (LPS) activity was determined with a Limulus amoebocyte lysate assay (Hycult Biotechnology). Serum LPS binding protein (hereafter, LBP) (Hycult Biotechnology), IgG antibody to LPS core (hereafter, EndocAb) (Hycult Biotechnology, Uden, the Netherlands) and soluble CD14 (here after sCD14) (R&D Systems, Abingdon, United Kingdom) levels were measured by ELISA. The detection limit for LPS, LBP, sCD14 and EndocAb are 0.04 EU/ml, 4.4 ng/ml, 0.125 ng/ml and 0.125 EndoCab Median units (EMU/ml) respectively. Since HDL cholesterol is the main factor involved in neutralization of endotoxemia, we used the LPS/HDL ratio as a functional measure of the LPS activity [14].

Determination of serum pancreatic, gut and adipocytokine hormone levels

The levels of insulin, glucagon, adiponectin and leptin were measured in the serum using Bio-Plex Pro™ Human Diabetes Assays kit (Biorad, Hercules, CA). The lowest detection limits were: Insulin- 8.31 pg/ml, Glucagon–3.41 pg/ml, Adiponectin–75.4 pg/ml and Leptin–42.44 pg/ml. The intra- and the inter-assay coefficients of variation was less than 5%. Insulin resistance was estimated using Homeostatic Model Assessment of insulin resistance (HOMA-IR) calculated as fasting insulin (μU/ml) X fasting glucose (mmol/l) / 22.5.

Estimation of serum TNF-α, IL-6 and IL-1β levels

The levels of TNF-α, IL-6 and IL-1β in the undiluted serum were measured using ELISA kits (Invitrogen, NY, USA). The lowest detection limits were: TNF-α-1.0 pg/ml, IL-6-0.1 pg/ml, IL-1β-0.01 pg/ml.

Statistical analysis

Data are expressed as geometric mean values. Student t-test was used to compare groups for continuous variables, whereas χ^2 test or Fisher exact test (as appropriate) was used to compare proportions. The Mann–Whitney U test was used in case of non-normally distributed parameters to compare means. Spearman correlation analysis was carried out to determine the association of LPS, EndoCab and sCD14 with the other risk variables. Multivariate Logistic regression analysis was used to determine the association of LPS, EndoCab and sCD14 with CAD. Kruskal-Wallis test was used for parameters that did not show normal distribution. Multiple comparisons were corrected using the Holm's correction for each set of analysis. All the analyses were done using SPSS statistical package (Version 20.0; SPSS, Chicago, IL) and P value less than 0.05 was considered significant.

Results

Table 1 shows the clinical and biochemical characteristics of the study subjects. Compared to the control subjects, the CAD subjects had increased levels of fasting plasma glucose (p=0.034), glycated hemoglobin (p=0.010), serum triglyceride levels (p=0.008), HOMA-IR (p=0.003) and hs-CRP (p=0.029). In the CAD group, 28.3% had obesity, 36.9% had hypertension, 58.7% had dyslipidemia and 29.5% had metabolic syndrome. Among the CAD subjects 10.8% were on statins and 23% were on hypertension drugs (11% on beta blockers, 6% on calcium antagonist and 6% on ACE inhibitors). In the control group 2% were on anti-hypertensive drugs (beta blockers).

To determine the association of microbial translocation related markers with CAD, the serum levels of LPS, LBP, sCD14 and EndoCab were compared between control and CAD subjects. As seen in Figure 1, the CAD subjects had significantly higher levels of LPS (Geometric mean (GM): CAD-45.83 vs. control-14.73EU/ml (5.6-1518.2EU/ml); p<0.001) (Figure 1a), LPS activity (p<0.001) (Table 1) and EndoCab

Parameters	Control (n=45)	CAD (n=46)	P
Age [years]	45.8 ± 15.0	46.1 ± 14.0	0.167
Gender (M)[%]	54.7	53.3	0.894
Obesity [%]	14.6	28.3	0.125
Hypertension [%]	4.8	36.9	<0.001
Fasting plasma glucose [mg/dl][a]	84.4 ± 6.6	95.30 ± 32.1	0.034
Glycated hemoglobin [%]	5.5 ± 0.5	6.1 ± 1.2	0.010
Diabetes [%]	-	-	NA
Serum total cholesterol [mg/dl][a]	174.1 ± 29.6	193.0 ± 59.6	0.066
Serum triglyceride [mg/dl][a]	85.0 ± 36.3	113.2 ± 57.1	0.008
High density Lipoprotein ((HDL) [mg/dl][a]	46.5 ± 8.6	45.7 ± 11.7	0.741
Low density Lipoprotein (LDL) [mg/dl][a]	110.0 ± 27.5	124.6 ± 54.1	0.136
Dyslipidemia [%]	42.9	58.7	0.138
HOMA-IR[a]	1.1 ± 0.6	1.7 ± 1.1	0.003
hs-CRP [mg/L] (geo mean)	1.28	3.24	0.029
LPS activity (geo mean)	0.21	1.01	<0.001
Smoking [%]	35	24	0.226
Metabolic syndrome [%]	2.4	29.5	0.001

[a]For FPG, STC, STL, HDL, LDL and HOMA-IR mean ± SD is reported

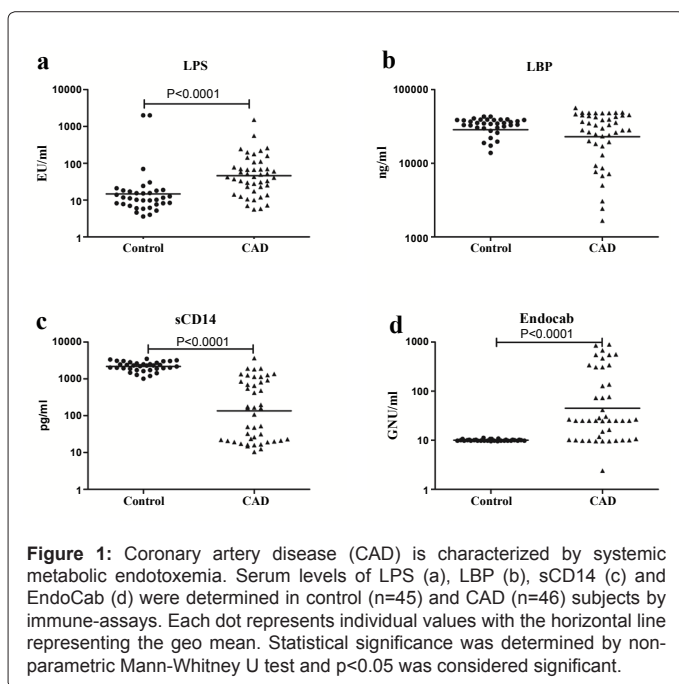**Table 1:** Clinical characteristics of the study subjects.

Figure 1: Coronary artery disease (CAD) is characterized by systemic metabolic endotoxemia. Serum levels of LPS (a), LBP (b), sCD14 (c) and EndoCab (d) were determined in control (n=45) and CAD (n=46) subjects by immune-assays. Each dot represents individual values with the horizontal line representing the geo mean. Statistical significance was determined by non-parametric Mann-Whitney U test and p<0.05 was considered significant.

correlation with sCD14. EndoCab showed strong positive association with hsCRP, HOMA-IR and a negative correlation with sCD14. sCD14 showed strong negative correlation with hsCRP, HOMA-IR and IMT. Logistic regression analysis showed an association of CAD only with EndoCab (β=1.35; 95% CI: 1.083-1.689; p=0.008) but not with LPS and sCD14.

Next, to elucidate the possible association of hormones with CAD, we measured serum glucagon, adiponectin and leptin levels in control and CAD subjects. As shown in Figure 3, significantly increased levels of glucagon (Geometric Mean (GM): CAD-13.15 vs. control-0.44 pg/ml (0.2-22.7 pg/ml); p<0.001) and decreased levels of adiponectin (CAD- 3151.1 vs. control- 4573 pg/ml (456.7-22,518pg/ml); p=0.0034) were seen in CAD subjects. Leptin levels showed no difference between control and CAD subjects. Next we carried out correlation analysis between the components of microbial translocation and serum hormones. As can be seen in Figure 4, glucagon showed a positive correlation with LPS (r=0.445; p<0.001) and EndoCab (r=0.699; p<0.001) and a negative correlation with sCD14 (r=-0.430; p<0.001). Adiponectin showed a negative correlation only with Endocab (r=-0.374; p=0.001). Correlation analysis between the serum hormones and the risk factors of CAD showed a good positive correlation of Glucagon with IR (r=0.318) and hsCRP (r=0.250), whereas there was no correlation with IMT (data not shown). Adiponectin did not show a correlation with any of the risk factors for CAD (data not shown). Significant increase in the levels of pro inflammatory cytokines TNF-α (Geometric Mean (GM): CAD-3.86 vs. control-2.22 pg/ml (1.010-1290.45 pg/ml); p=0.0056), IL-6 (CAD-3.54 vs. control-1.61 pg/ml (0.060-850.3 pg/ml); p=0.0165) and IL-1β (CAD- 1.53 vs. control-1.23 pg/ml (0.010-20.54 pg/ml); p=0.0266), was observed in the CAD group when compared to the control. Correlation analysis showed a good positive correlation of IL-1β with Endocab (r=0.245) and LPS (r=0.281). TNF-α showed a negative correlation with sCD14 (r=-0.232).

Discussion

In the present study, we attempted to delineate the link between EndoCab, LBP and sCD14 levels with chronic low-grade endotoxemia and tried to find an association of these factors with the major risk factors of CAD, including adiponectin and leptin levels. The major findings of the study are as follows: In CAD subjects 1. The serum LPS and EndoCab levels were significantly increased while the levels of sCD14 was decreased indicating endotoxemia, 2. While LPS and EndoCab levels showed good positive correlation with the major risk factors of CAD (IMT, hsCRP and HOMA-IR), sCD14 showed a negative association with these risk factors, 3. Significantly elevated levels of glucagon and decreased levels of adiponectin (but not leptin) were also observed in CAD group and 4. CAD subjects also had significantly high levels of TNF-α, IL-6 and IL-1β. The increased levels of glucagon along with increased HOMA-IR seen in CAD subjects might be responsible for the increased levels of fasting blood glucose and HbA1c.

Serum EndoCab levels serve as good prognostic marker for post-operative mortality rate associated acute inflammation as seen in septicemia and endotoxemia [4]. It is generally believed that, high levels of EndoCab neutralize LPS conferring protection against acute inflammation in the post-operative phase. But the significance of EndoCab levels under conditions of chronic inflammation is less well explored. Our study showed high levels of both EndoCab and LPS in CAD subjects compared with control, and the significance of this is difficult to predict at this juncture. Both IgG and IgM EndoCab levels become rapidly depleted during acute septicemia from different
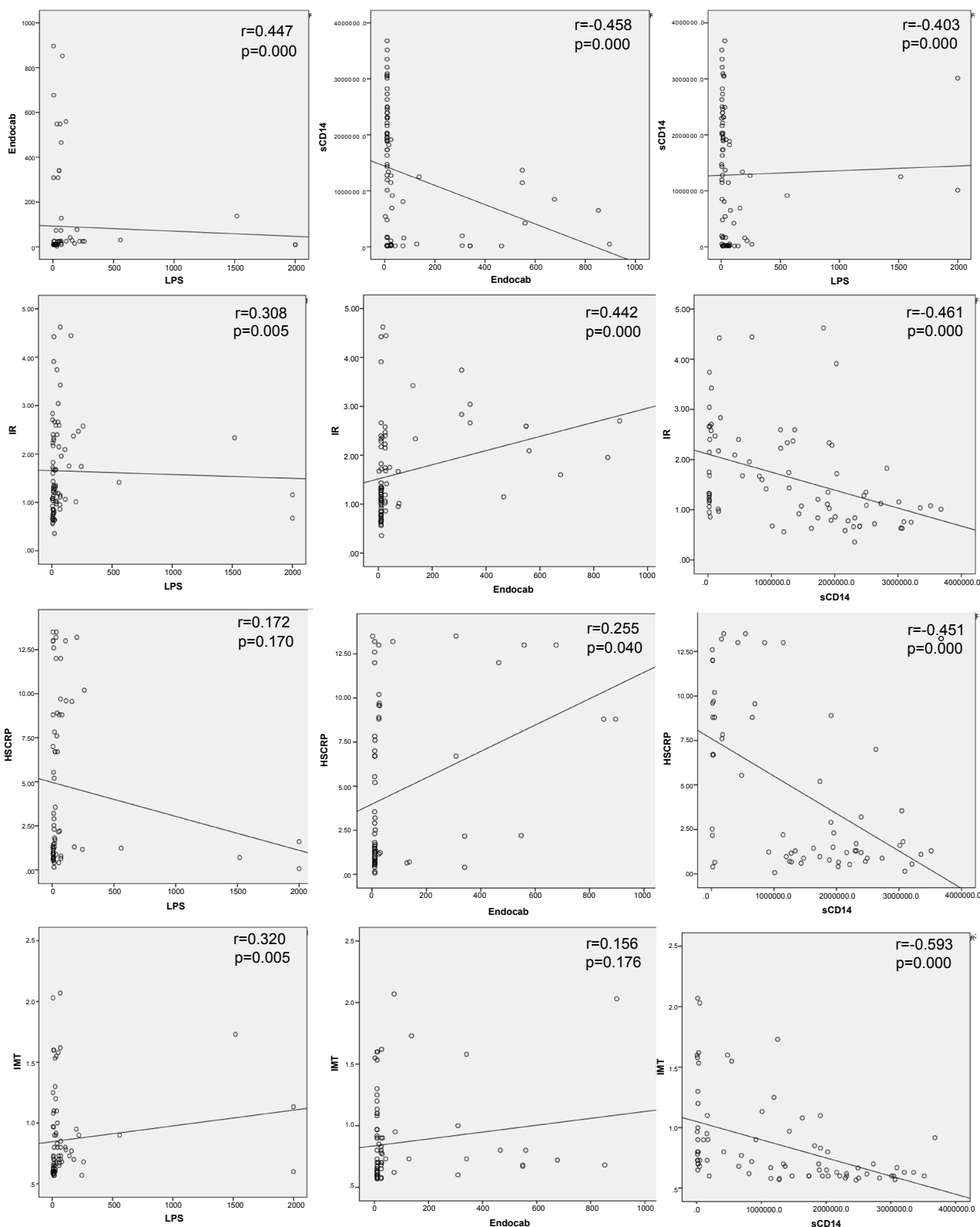
levels (CAD-44.75 vs. control-10.03GMU/ml (2.4-896.2 GMU/ml); p<0.001) (Figure 1d) and lower levels of sCD14 (CAD-135622 vs. control-2172382 ng/ml (10442.6-3679201 ng/ml); p<0.001) (Figure 1c). The LBP levels were not significantly different between the groups (Figure 1b).

Quartile analysis showed a significantly higher percentage of CAD subjects in the highest quartile of EndoCab (>26.6 EMU/ml) (CAD-24% vs. Controls 0%) and LPS (>65.05 EU/ml) (CAD-21% vs. Controls 4%), while a reverse trend was seen for sCD14 (>2198398.7 ng/ml) (CAD-1% vs. Control-24%). Next we carried out a correlation analysis to find the association of these biomarkers with the major risk factors of CAD. As can be seen in Figure 2, LPS showed strong positive correlation with EndoCab, HOMA-IR and IMT and a negative

Figure 2: Correlation analysis between components of microbial translocation and traditional risk factors of in subjects with Coronary Artery Disease (CAD). Plasma levels of lipopolysaccharide (LPS) were correlated with the EndoCab (a), IR (d), hsCRP (i) and IMT (l) in CAD subjects (n = 46). Plasma levels of endotoxin core antibody IgG (EndoCAb) were correlated with sCD14 (b), IR (e), CRP (j) and IMT (m). Plasma levels of sCD14 were correlated with LPS (c), IR (f), CRP (k) and IMT (n). P and r values were calculated using the Spearman rank correlation test at 95% confidence intervals.
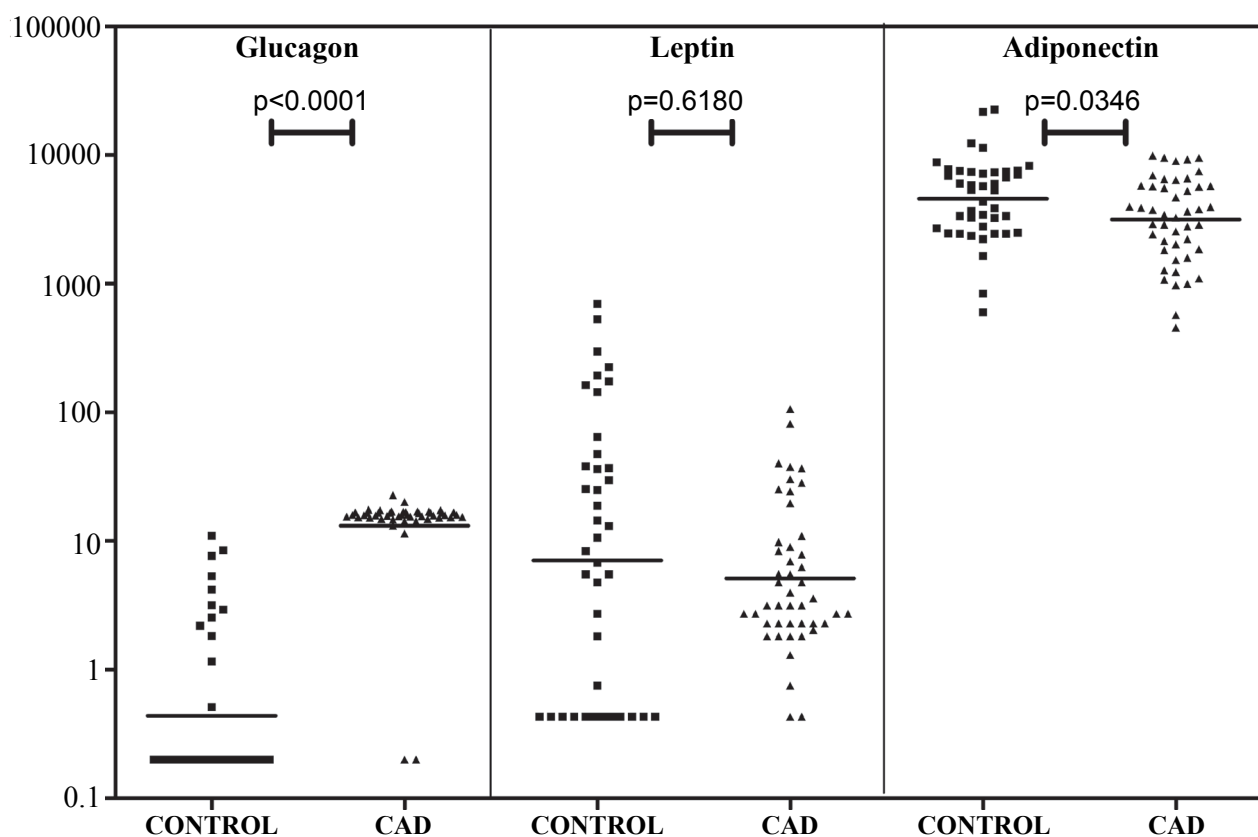
Figure 3: Coronary artery disease (CAD) is associated with high levels of glucagon and low levels of adiponectin. Serum levels of Glucagon (a), Leptin (b) and Adiponectin (c) were determined in control (n=45) and CAD subjects (n=46) by multiplex assay. Each dot represents individual values with the horizontal line representing the geo mean. Significance was calculated by non-parametric Mann-Whitney U test and p<0.05 was considered significant.

sources of sepsis [15]. However, the levels of IgG EndoCab increases in situations of chronic exposure to endotoxin as seen in conditions like inflammatory bowel disease [16], Crohn's disease [17], urinary tract infections with renal stones [18] and obstructive jaundice [19]. Apart from bacterial infections EndoCab levels were also found to be altered in certain viral and parasitic diseases [20]. Recently, increased levels of EndoCAb have been reported in hookworm infection [21], extra pulmonary TB [22] and African sleeping sickness [23]. The strong positive association seen between EndoCab with hsCRP and HOMA-IR indicates that these antibodies, by way of modulating the activity/levels of LPS can play a role in both inflammation as well as insulin resistance in CAD.

sCD14 has recently emerged as a major serum determinant in the prognosis of CAD [24]. Interestingly, sCD14 is able to mediate LPS-activation in CD14-negative cells such as endothelial and smooth muscle cells, events that are important for the development of atherosclerosis and its complications (like CAD) [25]. At higher concentrations, it downregulate LPS-induced responses by transferring LPS to lipoproteins followed by its removal [25]. Recently, in a genomewide association study, a novel haplotype of CD14 was reported, which was strongly associated with low levels of sCD14 and increased risk for cardiovascular disease among blacks [24]. It is of importance to note that even in this study sCD14 levels were significantly lower in CAD

subjects and showed negative correlation with hsCRP, HOMA-IR and IMT [24].

Even though the CAD group had twice the proportion of obese subjects compared to the control group, the serum leptin levels were not significantly different between these two groups. Previous studies have shown poor correlation between BMI and leptin levels in the south Indian population [26,27]. The significantly reduced level of adiponectin in the CAD group is in accordance with previous reports [28]. Adiponectin which is a major hormone that attenuates insulin resistance and promotes endothelial functioning was found to be lowered under conditions of metabolic diseases like obesity, diabetes and cardiovascular diseases [28].

The moderate endotoxemia with increased Endocab and decreased sCD14 together suggests sustained immune activation resulting in chronic inflammation. Several immune and non-immune cells express LPS receptor (TLR4) and respond to LPS stimulation by secreting pro-inflammatory cytokines which in turn reinforce the action of LPS creating a positive feed-back loop [29]. The end result is a systemic increase in the levels of IL-1β, IL-6, TNF-α as seen in the CAD subjects in this.

To summarize, chronic low-grade endotoxemia, as seen in the case of CAD, was associated with increased levels of EndoCab and decreased levels of sCD14 and was associated with chronic inflammation as seen
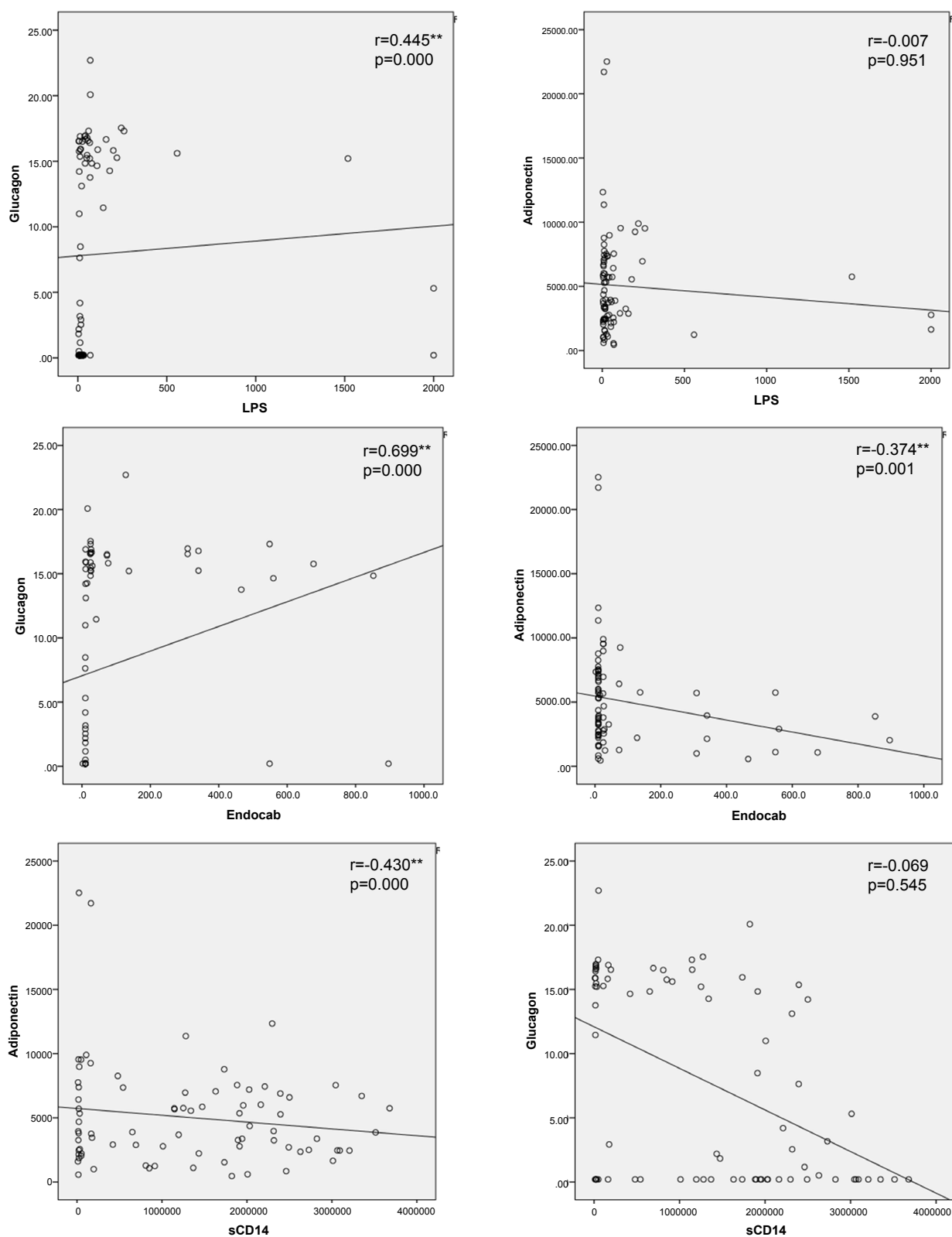
Figure 4: Correlation analysis between components of microbial translocation and serum hormone levels in CAD. Plasma levels of lipopolysaccharide (LPS) were correlated with the glucagon (a) and adiponectin (b) levels in CAD subjects (n = 46). Plasma levels of endotoxin core antibody IgG (EndoCAb) were correlated with glucagon (c) and adiponectin (d). Plasma levels of sCD14 were correlated with glucagon (e) and adiponectin (f). P and r values were calculated using the Spearman rank correlation test at 95% confidence intervals.

by the elevated levels of TNF-α, IL-6 and IL-1β. While hormones which counteract plaque formation (adiponectin) were decreased [30], those which promote hyperglycemia (glucagon) were significantly elevated. One of the limitations of this study is that, being cross-sectional in nature, no conclusions regarding a causal relationship between endotoxemia, inflammation and altered hormone secretion can be made. However the strength of this study is that, this is a population based study which systematically reviews components of microbial translocation in age and gender matched CAD subjects and that too in a high risk ethnic population. In summary, this study suggests that CAD in Asian Indians is characterized by high levels of EndoCab and low levels of sCD14 which might increase chronic inflammation leading to pathology.

Acknowledgments

Author contributions

Conceived and designed the experiments: VA VM SB. Performed the experiments: HM. Analyzed the data: VA HM and VS. Wrote the paper: VA.

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