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IMPAIRED VASCULAR ENDOTHELIAL GROWTH FACTOR-A AND INFLAMMATION IN SUBJECTS WITH PERIPHERAL ARTERY DISEASE

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Abstract

We compared apoptosis, cellular oxidative stress, and inflammation of cultured endothelial cells treated with sera from 130 subjects with peripheral artery disease (PAD) and 36 control subjects with high burden of co-morbid conditions and cardiovascular risk factors. Secondly, we compared circulating inflammatory, antioxidant capacity, and vascular biomarkers between the groups. The groups were not significantly different ($p>0.05$) on apoptosis, hydrogen peroxide, hydroxyl radical antioxidant capacity, and nuclear factor k-light-chain-enhancer of activated B cells. Circulating tissue necrosis factor alpha (TNFα) ($p=0.016$) and interleukin-8 ($p=0.006$) were higher in the PAD group, whereas vascular endothelial growth factor-A (VEGF-A) ($p=0.023$) was lower. PAD does not impair the endothelium beyond that which already occurs from co-morbid conditions and cardiovascular risk factors in subjects with claudication. However, subjects with PAD have lower circulating VEGF-A than controls, and higher circulating inflammatory parameters of TNFα and IL-8.

Keywords

Claudication; Inflammation; Peripheral Artery Disease; Vascular Endothelial Growth Factor-A

INTRODUCTION

Peripheral artery disease (PAD) is prevalent in 8 million men and women in the United States,[1] and in more than 12% of community dwelling people aged 65 years.[2] PAD is

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Declaration of interest

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associated with increased prevalence of diseases in the coronary, cerebral and renal arteries.[2, 3] More than 60% of those with PAD have concomitant cardiovascular and/or cerebrovascular disease,[3] thereby contributing to their elevated rates of cardiovascular mortality.[4, 5] The cost associated with PAD is comparable to, if not higher than cardiac dysrhythmias, congestive heart failure and cerebrovascular disease, averaging $3.9 billion for total Medicare paid PAD-related care annually.[6] Many of those with PAD are physically limited by ambulatory leg pain, resulting in ambulatory dysfunction,[7, 8] impaired physical function,[9, 10] lower physical activity levels,.[11, 12] and even worse health-related quality of life scores than individuals with coronary artery disease and congestive heart failure.[13] Furthermore, PAD subjects have increased rates of functional decline and mobility loss compared to those without PAD.[9, 10, 14]

Endothelial dysfunction is an early marker of vascular dysfunction prior to the development of structural changes and clinical symptoms,[15–21] contributes to the progression of atherosclerosis,[21] and increases the risk of coronary events.[15, 17, 21–23] We[24] and others[21] have found that endothelial function is impaired in PAD subjects. Furthermore, co-morbid conditions and cardiovascular risk factors are highly prevalent in subjects with PAD, such as metabolic syndrome,[25] diabetes,[2, 3] hypertension,[2, 3] dyslipidemia,[2, 3] smoking,[2, 3] ambulatory dysfunction[7, 8] and low physical activity levels.[11, 12] The greater co-morbid burden in PAD subjects contributes to higher levels of inflammation[26] and oxidative stress,[27] but it is not clear whether these co-morbid conditions exert additional influence on inflammatory and vascular biomarkers beyond that seen from the atherosclerotic burden of PAD. Thus, we believe that a control group with a high burden of co-morbid conditions is appropriate to compare with patients with PAD.

The purpose of this study was to compare apoptosis, cellular oxidative stress, and inflammation of cultured endothelial cells treated with sera from subjects with PAD and control subjects with high burden of co-morbid conditions and cardiovascular risk factors. A second aim was to compare circulating inflammatory, antioxidant capacity, and vascular biomarkers between the 2 groups. We hypothesized that subjects with PAD would have greater endothelial apoptosis, cellular oxidative stress, inflammation, and lower antioxidant capacity than controls, and worse systemic inflammatory and vascular biomarkers.

METHODS

Subjects

Approval and Informed Consent—The procedures used in this study were approved by the institutional review board at the University of Oklahoma Health Sciences Center (HSC) and by the Research and Development committee at the Oklahoma City VA Medical Center. Written informed consent was obtained from each patient at the beginning of investigation.

Recruitment—Subjects were recruited by referrals from vascular labs and vascular clinics from the University of Oklahoma HSC and the Oklahoma City VA Medical Center for possible enrollment into a randomized controlled exercise rehabilitation study for the treatment of leg pain secondary to PAD.[28] The data and analyses for this study were part of the baseline assessments obtained for the exercise study.

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Medical Screening through History and Physical Examination

Subjects were evaluated at the Clinical Research Center (CRC), at the University of Oklahoma HSC. Subjects arrived at the CRC in the morning fasted, but were permitted to take their usual morning medication. Demographic information, height, weight, waist circumference,[29] cardiovascular risk factors, co-morbid conditions, claudication history, blood samples, and a list of current medications were obtained from a medical history and physical examination at the beginning of the study. All subjects lived independently at home.

Inclusion and Exclusion Criteria for the PAD Group—Subjects with intermittent claudication were included in this study if they met the following criteria: (a) a history of ambulatory leg pain, (b) ambulation during a graded treadmill test limited by leg pain consistent with intermittent claudication,[7] and, (c) an ankle-brachial index (ABI) 0.90 at rest[2] or an ABI 0.73 after exercise because some subjects with PAD have normal values at rest which only become abnormal following an exercise test.[30] Subjects were excluded from the PAD group for the following conditions: (a) absence of PAD (ABI > 0.90 at rest and ABI > 0.73 after exercise), (b) inability to obtain an ABI measure due to non-compressible vessels, (c) asymptomatic PAD determined from the medical screening, ABI test, and graded treadmill test, (d) use of medications indicated for the treatment of claudication (cilostazol and pentoxifylline) initiated within 3 months prior to investigation, (e) exercise tolerance limited by any disease process other than PAD, (f) active cancer, (g) end stage renal disease defined as stage 5 chronic kidney disease, and, (h) abnormal liver function. A consecutive series of 184 individuals were evaluated for eligibility, and 130 subjects were deemed eligible for inclusion in the PAD group and 54 subjects were ineligible.

Inclusion and Exclusion Criteria for the Control Group—The 54 subjects who were excluded from the PAD Group were considered for inclusion into the control group if they did not have PAD according to the following criteria: (a) ambulation during a graded treadmill test was not limited by leg pain consistent with intermittent claudication,[7] and, (b) an ABI > 0.90 at rest[2] and an ABI > 0.73 after exercise.[30] These subjects were excluded from the control group if they met any of the following conditions: (a) presence of PAD (ABI 0.90 at rest and ABI 0.73 after exercise), (b) inability to obtain an ABI measure due to non-compressible vessels, (c) active cancer, (d) end stage renal disease defined as stage 5 chronic kidney disease, and, (e) abnormal liver function. Of the 54 subjects who were considered for inclusion into the control group, 36 subjects were deemed eligible and 18 subjects were ineligible.

Measurements

Graded Treadmill Test: Claudication Onset Time (COT), peak walking time (PWT), ABI, and Ischemic Window

COT and PWT: Subjects performed a progressive, graded treadmill protocol to determine study eligibility, as well as to obtain outcome measures related to peak exercise performance.[7] The COT, measured as the walking time at which the patient first experienced pain, and the PWT, measured as the walking time at which ambulation could not continue

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due to maximal pain, were both recorded to quantify the severity of claudication. Using these procedures, the test-retest intraclass reliability coefficient is R = 0.89 for COT,[7] and R = 0.93 for PWT.[7]

ABI and Ischemic Window: ABI measures were obtained from the more severely diseased lower extremity during supine rest and 1, 3, 5, and 7 min after the treadmill test.[7, 31] The reduction in ankle systolic blood pressure after treadmill exercise from the resting baseline value was quantified by calculating the area under the curve, referred to as the ischemic window.[32] Because the ischemic window is a function of both PAD severity and the amount of exercise performed, the ischemic window was normalized per meter walked.

Blood Sampling: Venipuncture was done to obtain the blood specimen from an antecubital vein. The blood was collected in vacutainers and then distributed in 0.5 ml aliquots. The samples were stored at −80°C, and were subsequently batched for analysis.

Endothelial Cell Cultures: We used a cell culture-based bioassay approach utilizing cultured primary human arterial endothelial cells to characterize the endothelial effects of circulating factors present in the sera of PAD subjects. In brief, endothelial cells (purchased from Cell Applications, Inc., San Diego, CA, after passage 4; age of the donors is unknown) were initially cultured in MesoEndo Endothelial Cell Growth Medium (Cell Applications, Inc) followed by Endothelial Basal Medium supplemented with 10% fetal calf serum until the time of serum treatment, as described.[33–36] For treatment, fetal calf serum was replaced with serum (10%; for 24–48 h) collected from our subjects, following our published protocols with modifications.[37] Cells cultured in Endothelial Basal Medium supplemented with 10% fetal calf serum served as an additional control.

Apoptosis Assay: Cultured endothelial cells were treated with sera from PAD subjects and their respective controls (for 24 h). To determine whether circulating factors present in the sera of PAD subjects exert pro-apoptotic effects, apoptotic cell death was assessed by measuring caspase activities using Caspase-Glo 3/7 assay kit (Promega, Madison, WI) as previously reported.[38, 39] In 96-well plates 50 μl sample was mixed for 30 sec with 50 μl Caspase-Glo 3/7 reagent and incubated for 2 h at room temperature. Lyses buffer with the reagent served as blank. Luminescence of the samples was measured using an Infinite M200 plate reader (Tecan, Research Triangle Park, NC). Luminescent intensity values were normalized to the sample protein concentration and are expressed as relative changes.

Cellular Reactive Oxygen Species (ROS) Production: To assess cellular oxidative stress induced by factors present in the sera, hydrogen peroxide (H_2O_2) production in detector endothelial cells was measured fluorometrically using the Amplex Red/horseradish peroxidase assay as described.[40, 41] The rate of H_2O_2 generation was assessed by measuring resorufin fluorescence for 60 min by a Tecan Infinite M200 plate reader. A calibration curve was constructed using H_2O_2 and the production of H_2O_2 in the samples was calculated as pmol H_2O_2 released per minute. Hoechst 33258 fluorescence, representing cellular DNA content, was used for normalization. Data are expressed as arbitrary units.

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Transient Transfection, NF-κB Reporter Gene Assay: To assess cellular pro-inflammatory effects induced by factors present in the sera, transcriptional activity of NF-κB was tested in serum-treated detector endothelial cells by a reporter gene assay as described.[42] We used a NF-κB reporter comprised of a NF-κB response element upstream of firefly luciferase (NF-κB-Luc, Stratagene) and a renilla luciferase plasmid under the control of the cytomegalovirus promoter (as an internal control). Transfections in endothelial cells were performed using the Amaxa Nucleofector technology (Amaxa, Gaithersburg, MD), as we have previously reported.[42] Firefly and renilla luciferase activities were assessed after 24 h using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) and a Tecan Infinite M200 plate reader.

Serum Antioxidant Capacity: To compare the capacity of antioxidant enzymes and other redox molecules present in the sera of PAD subjects to counterbalance the deleterious effects of oxidative stress, we assessed the Hydroxyl Radical Antioxidant Capacity (HORAC) using the OxiSelect HORAC Activity Assay (Cell Biolabs Inc., San Diego, CA) as previously described.[38] The HORAC Activity Assay is based on the oxidation-mediated quenching of a fluorescent probe by hydroxyl radicals produced by a hydroxyl radical initiator and Fenton reagent. Antioxidants present in the sera delays the quenching of the fluorescent probe until the antioxidant activity in the sample is depleted. The antioxidant capacity of the sera was calculated on the basis of the area under the fluorescence decay curve, compared to an antioxidant standard curve obtained with gallic acid. Data are expressed as arbitrary units.

Circulating inflammatory and vascular biomarkers: Assessment of circulating cytokines and biomarkers present in the sera was performed using a Millipex Human Adipokine Magnetic Bead Kit for tissue necrosis factor alpha (TNF α), interleukin-1b (IL-1b), interleukin-6 (IL-6), interleukin-8 (IL-8), monocyte chemotactic protein-1 (MCP-1), hepatocyte growth factor (HGF), and nerve growth factor (NGF). A Millipex Human Cardiovascular Disease (CVD) Panel 1 Kit was used for myeloperoxidase (MPO), matrix metallopeptidase 9 (MMP-9), E selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), and plasminogen activity inhibitor-1 (PAI-1). A Millipex Human Apolipoprotein Kit was used for apolipoprotein B and apolipoprotein CIII. The Millipore kits were purchased from the EMD Millipore, Billerica, MA. Affymetrix Procarta Immunoassay was used for the detection of serum amyloid A (SAA), vascular endothelial growth factor-A (VEGF-A) and adiponectin. These assays were performed according to the manufacturer’s protocols. Sample protein content was determined for normalization purposes by a spectrophotometric quantification method using BCA reagent (pierce Chemical Co., Rockford, IL).

High-Sensitivity C-Reactive Protein (hsCRP): A high-sensitivity Near Infrared Particles Immunoassay was used to quantify the concentration of hsCRP from a serum sample of 300 μl, the optimum sample volume for this specific assay. A commercially available device, the SYNCHRON LX-20 (Beckman-Coulter; California, USA), was used to automatically perform the assay. Prior to performing each assay, the SYNCHRON system was calibrated,

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and a calibration curve was established. The "normal" reference range for concentrations of hsCRP using this high-sensitivity assay is 0.0 – 3.3 mg/L.[43]

Oxidized LDL: Plasma oxidized LDL was measured by immunoassay (Mercodia, Uppsala, Sweden) according to the manufacturer's protocol. For oxidized LDL, average intra-assay precision is 5% and inter-assay precision is 8.7%.

Statistical Analyses

Clinical characteristics of subjects were examined for differences between subjects with PAD and controls using independent t-tests for measurement variables and one degree of freedom Chi square tests for dichotomous variables. Preliminary examination of response variables revealed large deviations from normal distribution and, therefore, statistical methods not based on this assumption were used for hypothesis testing. Although there is a significant difference between the subjects with PAD and the controls for sex and current smoking, the differences would affect comparison of the groups only if the response variable is associated with either sex or current smoking. The Spearman partial correlation of current smoking controlling for PAD is significant for only leptin, adiponectin and VCAM-1. The Spearman partial correlation of sex within current smokers controlling for PAD was not significant for any variable. Corresponding correlation within non-smokers is significant for only one response variable, leptin. For each variable with no significant association with either sex or current smoking, values were summarized within both PAD and control groups by presenting median and interquartile range, and 2 groups were compared with Wilcoxon test. For variables with only significant association with current smoking, separate summaries and comparisons were made for smokers and non-smokers. For leptin, which had significant association with sex and within non-smokers, summaries and comparisons were made for each of the sex/non-smoking groups and for the current smokers with sexes combined.

RESULTS

The clinical characteristics of subjects with PAD and controls are displayed in Table 1. By definition, the PAD group had a lower ABI ($p < 0.001$) than the control group. Additionally, the PAD group had a higher percentage of men ($p = 0.044$) and a higher prevalence of current smoking ($p = 0.008$) than the controls. The 2 groups were similar on the remaining variables ($p > 0.05$), but there were trends for the PAD group having a higher prevalence of hypertension ($p = 0.060$) and dyslipidemia ($p = 0.060$), and for the control group having a higher prevalence of arthritis ($p = 0.087$).

The measurements from cultured endothelial cells treated with sera from subjects with PAD and controls are shown in Table 2. The 2 groups were not significantly different on apoptosis, H_2O_2, HORAC, and NF-κB. The circulating inflammatory and vascular biomarkers of subjects with PAD and controls are displayed in Table 3. TNFα ($p = 0.016$) and IL-8 ($p = 0.006$) were higher in the PAD group than in the controls, whereas VEGF-A ($p = 0.023$) was lower in the PAD group, and there was a trend for VCAM-1 to be higher in subjects with PAD who were non-smokers than in controls who were non-smokers (p =

0.053). The PAD and control groups were not significantly different on the remaining variables ($p > 0.05$).

DISCUSSION

The primary finding was that the PAD group did not have worse endothelial measures than a control group with high burden of co-morbid conditions and cardiovascular risk factors. However, the PAD group had lower circulating VEGF-A than the controls, and higher circulating inflammatory markers of TNFα and IL-8.

Subjects with Claudication Do Not Have Worse Endothelial Measures than Controls

A novel aspect to the current investigation was that endothelial measures consisting of apoptosis, cellular ROS production, serum antioxidant capacity, and NF-κB were not statistically different between subjects with claudication and controls with similarly high prevalence of co-morbid conditions and cardiovascular risk factors. This finding supports our previous study which assessed endothelial function using the technique of brachial artery flow-mediated dilation in subjects with and without PAD.[24] The significant and negative predictors of brachial artery flow-mediated dilation were systolic blood pressure, fasting glucose, and the low-density to high-density lipoprotein cholesterol ratio, whereas ABI was not a significant predictor.[24] Collectively, our current and previous studies indicate that cardiovascular risk factors, particularly those related to metabolic syndrome, are more strongly associated with endothelial function than PAD. Thus, treatment of cardiovascular risk factors through medical management or with exercise training[44] may improve endothelial measures independent from the presence of PAD and claudication.

Greater Inflammation and Lower VEGF-A in Subjects with Claudication

Another primary finding in the current study was that the subjects with PAD and claudication had greater inflammation, as measured by TNFα and IL-8 cytokines, than compared to controls with high cardiovascular and co-morbid burden. This supports previous work demonstrating that TNFα and IL-6 cytokines were higher in subjects with PAD compared with healthy controls,[26] as well as IL-8.[45] In addition to these established inflammatory markers, high-density lipoprotein cholesterol (HDL-C) may also influence inflammation. HDL-C was obtained in this study to better characterize the groups for dyslipidemia, but was not considered in the analyses of inflammatory measures because we have previously found that HDL-C was not different between patients with PAD and control subjects.[46] Overall, the observation that inflammatory measures are elevated in patients with PAD emphasizes the need to develop interventions that will reduce inflammation. We have recently shown that higher levels of community-based, daily ambulatory activity are associated with lower levels of inflammation in patients with PAD and claudication,[47] suggesting that an exercise intervention program may be efficacious to treat inflammation in this population. Furthermore, the efficacy of pharmacologic therapy, such as statin medications, cilostazol, and other medications to favorably alter inflammation of patients with PAD and claudication should be better established.

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One novel aspect of the current investigation was the use of a control group that was not healthy, enabling us to better distinguish between the influence of PAD on inflammatory biomarkers from the influence of cardiovascular risk factors. Interestingly, the previous finding of higher levels of adhesion molecules and selectins in subjects with PAD compared to healthy controls[26] was not confirmed in the current study. This suggests that adhesion molecules and selectins are elevated with PAD primarily due to the concomitant cardiovascular risk factors rather than to the atherosclerotic burden of PAD.

Another novel aspect to the current investigation was the finding that VEGF-A is lower in subjects with PAD and claudication compared to controls. VEGF-A is an angiogenic growth factor, and is positively associated with capillary number.[48–51] The lower level of VEGF-A in the subjects with PAD and claudication suggests that they have lower levels of angiogenesis, which supports previous work demonstrating that lower VEGF-A is associated with a lower capillary/fiber ratio in subjects with PAD.[52]

There are limitations to this study. A self-selection bias may exist regarding study participation, as subjects who participated in this trial were volunteers. Therefore, they may represent those who were more interested in participation, who had better access to transportation to the research center, and who had relatively better health than PAD subjects who did not volunteer. Furthermore, the results of this study are only applicable to PAD subjects who are limited by claudication, and may not be generalized to subjects with less severe or more severe PAD. However, women and African-Americans are well represented, and typical risk factors for PAD such as smoking, diabetes, hypertension, dyslipidemia, and obesity are highly prevalent. Thus, in subjects with PAD and claudication, the findings of the present study are generalizable to the large proportion of men and women with PAD and in controls who have numerous co-morbid conditions. Finally, there are limitations associated with the design of the study. The comparison of the measures between the PAD group and the control group utilizes a cross-sectional design, which does not allow causality to be established.

In conclusion, PAD does not impair the endothelium beyond that which already occurs from co-morbid conditions and cardiovascular risk factors in subjects with claudication. However, subjects with PAD have lower circulating VEGF-A than controls, and higher circulating inflammatory parameters of TNFα and IL-8. The clinical implication is that optimal medical management of cardiovascular risk factors in subjects with PAD and claudication may be an efficacious approach to improve systemic inflammation, as well as vascular and metabolic biomarkers.

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Table 1

Clinical characteristics of subjects with peripheral artery disease (PAD) and controls. Values are means (standard deviation) or percentage of subjects in each group.

Variables	Control Group (n = 36)	PAD Group (n = 130)	P
Age (years)	63 (12)	64 (10)	0.694
Weight (kg)	88.1 (23.0)	84.5 (19.6)	0.350
Body Mass Index	31.5 (8.7)	29.6 (6.1)	0.137
Ankle/Brachial Index	1.17 (0.12)	0.72 (0.25)	< 0.001
Claudication Onset Time (sec)	-----	177 (146)	-----
Peak Walking Time (sec)	-----	394 (265)	-----
Ischemic Window (mmHg × min / meter)	-----	−0.65 (1.22)	-----
Sex (% Men)	33	52	0.044
Race (% Caucasian)	51	51	0.945
Current Smoking (% yes)	15	39	0.008
Hypertension (%)	71	85	0.060
Dyslipidemia (% yes)	65	80	0.060
Diabetes (%)	35	42	0.512
Obesity (% yes)	47	45	0.846
Myocardial Infarction (% yes)	15	19	0.600
Angina (% yes)	24	22	0.880
Cerebrovascular Accident (% yes)	12	18	0.359
Chronic Obstructive Pulmonary Disease (% yes)	21	25	0.565
Dyspnea (% yes)	44	58	0.159
Renal Disease (% yes)	9	5	0.340
Arthritis (% yes)	79	64	0.087

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Table 2

Measurements from cultured endothelial cells treated with sera from subjects with peripheral artery disease (PAD) and controls. Values are medians (interquartile ranges).

Variables	Control Group (n = 36)	PAD Group (n = 130)	P
Apoptosis (AU)	1.07 (0.72)	1.11 (0.36)	0.463
Cellular ROS production (AU)	28.28 (8.36)	27.50 (8.68)	0.578
NF-κB activity (AU)	1.53 (0.90)	1.27 (0.92)	0.175

ROS = reactive oxygen species.

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Table 3

Circulating inflammatory, antioxidant capacity, and vascular biomarkers of subjects with peripheral artery disease (PAD) and controls. Values are medians (interquartile ranges).

Variables	Control Group (n = 36)	PAD Group (n = 130)	P
C-Reactive Protein (mg/L)	2.71 (5.38)	3.70 (4.88)	0.544
Tumor necrosis factor alpha (pg/ml)	42.5 (22.5)	50.3 (25.5)	0.016
Interleukin-1b (pg/ml)	16.0 (5.8)	15.8 (5.1)	0.618
Interleukin-6 (pg/ml)	21.0 (13.8)	23.0 (12.9)	0.246
Interleukin-8 (pg/ml)	77.5 (46.8)	94.0 (61.5)	0.006
Monocyte chemotactic protein-1 (pg/ml)	1121.0 (1395.3)	1266.0 (1870.0)	0.392
Myeloperoxidase (pg/ml)	20.5 (27.8)	26.5 (40.3)	0.207
Matrix metallopeptidase 9 (pg/ml)	603.0 (336.0)	684.0 (313.3)	0.108
Serum Amyloid A (pg/ml)	9290.0 (3073.0)	9320.0 (3181.6)	0.612
Oxidized Low Density Lipoprotein (U/L)	72.2 (22.5)	69.8 (34.4)	0.073
Hydroxyl Radical Antioxidant Capacity (AU)	0.93 (0.21)	0.95 (0.23)	0.731
E selectin (pg/ml)	45.0 (73.8)	40.0 (43.5)	0.388
Vascular Cell Adhesion Molecule-1 (pg/ml)			
Smokers	1967.0 (593.0)	1963.3 (739.8)	0.953
Non-Smokers	2052.0 (805.0)	2242.0 (927.0)	0.053
Intercellular Cell Adhesion Molecule-1 (pg/ml)	1933.5 (1213.0)	1838.0 (1354.5)	0.916
Vascular Endothelial Growth Factor-A (pg/ml)	34.5 (106.0)	27.0 (30.9)	0.023
Leptin (pg/ml)			
Smokers	790.0 (3139.5)	1019.0 (2039.0)	0.884
Male Non-Smokers	1883.0 (1854.8)	1259.0 (1836.5)	0.251
Female Non-Smokers	5489.0(3493.9)	3903.3 (4220.3)	0.338
Adiponectin (pg/ml)			
Smokers	4878 (1272)	5263.5 (1850)	0.219
Non-Smokers	5674 (1741.8)	5641 (1092.5)	0.876
Plasminogen Activator Inhibitor-1 (ng/ml)	672.0 (494.3)	715.0 (482.3)	0.535
Apolipoprotein B (ng/ml)	57.0 (63.8)	63.0 (60.0)	0.611
Apolipoprotein CIII (ng/ml)	1417.0 (732.5)	1273.5 (947.5)	0.231
Hepatocyte Growth Factor (pg/ml)	67.0 (48.3)	67.0 (37.0)	0.962
Nerve Growth Factor (pg/ml)	13.0 (5.8)	14.0 (7.0)	0.189