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A Pilot Study on Co-relation between Inflammatory Markers with Ankle Brachial Index in Patients with Type 2 Diabetes in North Indian Population.

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***Abstract*—An increasing body of evidence supports the concept that inflammation plays a major role in the development and progression of atherosclerosis which in lower extremities leads to peripheral vascular disease. We hypothesized that inflammatory markers, WBCs, neutrophils, IL-6 and TNF-α would be associated with ABI, a hallmark of atherosclerotic burden.**

Research designs and methods: Among 60 type 2 diabetic patients, 35 were diagnosed for PVD (ABI ≤0.9), mean age 51.58±11.38 yrs, and 25 were without PVD (ABI >0.9), mean age 54.66±6.52 yrs. Serum concentrations of IL-6 and TNF-α were determined by enzyme linked immuno-sorbent assay.

Results: ABI was lower in PVD patients then without PVD (0.677±0.13 v/s 1.27±0.41, p<0.001, respectively). By linear regression analysis, TNF-α and IL-6 were significantly co-related with ABI in an inverse manner (β= -0.006, p= 0.030, β= -0.005, p= 0.018) respectively. There was no significant co-relation of ABI with WBCs and neutrophils.

Conclusion: ABI was strongly associated with PVD in type 2 diabetic patients. This study demonstrated that ABI was associated with IL-6 and TNF-α concentration with an inverse pattern making them prominent markers of inflammatory cascade involved in atherosclerosis.

***Index Terms*—Ankle Brachial Index, Inflammatory Markers, Peripheral Vascular Disease.**

I. INTRODUCTION

Peripheral vascular disease (PVD) is clinically defined by the absence of pedal pulses and/ or claudication in lower extremities [1]. Diabetes is an important risk factor for arterial disease with hypertension; smoking and hyperlipidemia making an additional contribute [2].

Ankle-brachial index (ABI) is a simple and non-invasive tool to document peripheral atherosclerosis. A declined value of ABI (<0.9) is a marker for the presence and progressive atherogenesis in lower limbs [3]. Narrowing of arteries with circulatory blood failure in lower extremities leads to non-healing ulcers with majority of them undergoing amputations making them one of the major causes of morbidity and mortality associated with type 2 diabetes. Atherosclerosis is a low grade chronic inflammatory disease [4], hence several candidate inflammatory mediators are found to play role as pro-atherogenic agents through inflammatory cascade [5]. Previous studies have reported altered values of blood inflammatory markers in atherosclerotic disorders. Loss of elasticity in inflamed arteries due to peripheral vascular disease, results in alteration in level of blood inflammatory markers including tumor necrosis factor alpha (TNF α), interleukin-6 (IL-6), white blood cell (WBC) count, and neutrophils [6]. Various case controls, epidemiological prospective studies quoted WBC count as independent marker of inflammation associated cardiovascular disease (CVD) [7]. In vascular diseases activated leukocytes emigrate, adhere to the endothelium and migrate through the arterial wall, resulting in the transfer of macrophages rich in oxidized lipoproteins that trigger of onset of inflamed atherosclerotic plaque formation. Accumulations of inflammatory cytokines including TNF-α, IL-6 and leukocytes are prominent in arterial plaques and may be considered as strong predictors of PVDs [8, 9]. IL-6 and TNF-α are major pro-inflammatory cytokine associated with initiation of acute phase response [10, 11]. But relation of ABI with these markers remains unclear. Clinical studies on association between index of PVD and inflammatory agents need to be more deeply evaluated, since it can predict the pivotal involvement of inflammatory markers with increased atherosclerotic burden in lower extremities. The present study was undertaken in type 2 diabetic patients to investigate whether ABI is associated with multiple blood inflammatory markers such as WBCs, neutrophils and cytokines TNF-α and IL-6.

II. RESEARCH DESIGNS AND METHODS

This was a pilot study conducted on 60 type 2 diabetes mellitus patients (45 men and 15 women) who were admitted in the Rajiv Gandhi Centre for Diabetes and Endocrinology, of Jawaharlal Nehru Medical College Hospital, Aligarh Muslim University, Aligarh. Subjects were diagnosed for type 2 diabetes mellitus according to criteria of World Health Organization. Patients with infectious, autoimmune, rheumatic and hematological diseases and severe renal or liver failure, as well as cancer were excluded from this study. A detailed history and physical examination was done of the subjects enrolled. Patients were evaluated for age, body mass index (BMI), duration of diabetes, lipid profile including total cholesterol (TC) triglycerides (TAGs), high density

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lipoprotein (HDL) and low density lipoprotein (LDL), glycated hemoglobin (HbA1c) prior the hospital stay, and cell blood count on the day of admission.

Twenty one patients were suffering from hyper cholestremia which was defined total serum cholesterol ≥150 mg/dl, and thirteen were suffering from hypertriglyceridemia as serum triglyceride was found to be ≥200 mg/dl on the basis of ADA-2010 criteria. Seventeen subjects were hypertensive with a systolic blood pressure (BP) ≥140 mm Hg and/or diastolic blood pressure (BP) ≥90 mm Hg. Informed consent was given by all patients and clearance was obtained from Institutional Ethics Committee (IEC) and Bio Ethical Committee (BEC).

A. Diagnosis of PVD

PVD was diagnosed on the basis of ABI value <0.9 in either of the lower extremity. For assessment patient was allowed to be in supine position for 20 minutes of acclimatization at room temperature before taking systolic blood pressure. For measurement of ankle pressure, occluding cuffs were placed just above the malleoli. The right and left ABI were automatically calculated by the device (Haedeco Doppler, Japan) by dividing the higher pressure on the dorsal pedal or posterior tibial artery of right and left foot, by the higher brachial pressure on either side. The smaller ABI value of either left or right foot was used for diagnosing PVD and data analysis.

B. Sample Collection and Analysis

Venous blood samples were collected after an overnight fast and centrifuged at 3000 rpm for 5 minutes at 4o C. Serum and plasma were immediately separated and stored in aliquots -80oC till further analysis. Plasma levels of IL-6 were determined by immune-enzymatic ELISA method Ani Biotech Oy, Orgenium Laboratories, Finland) with intra- and inter assay coefficient of 7.4 and 6.5 respectively. Serum concentration of TNF-α was measured by immuno-enzymatic ELISA method (Ani Biotech Oy, Orgenium Laboratories, Finland) with intra and inter assay coefficient of 6.8 and 6.2 respectively. White blood cell count and neutrophil percentage in serum samples was estimated by hematology analyzer.

C. Statistical analysis

Data are expressed as the mean ± SD and p value <0.05 was considered as statistically significant. Baseline characteristics between the groups with and without PVD were analyzed by a Student's t test. The correlation of markers of inflammation with conventional risk factors and the ABI was assessed by Pearson test. Relationship between ABI and clinical variables including inflammatory markers was determined by linear regression analysis. A P value <0.05 was accepted as indicating statistical significance. Independent association of clinical variable and markers of inflammation was analyzed by stepwise multivariate analysis. Statistical analyses were carried out using Medcalc software version 15.11.4.

III. RESULTS

Patients were divided into two groups on the presence or absence of PVD. Clinical and demographical characteristics of subjects in both groups are shown in (Table 1). There was no significant difference in age, HbA1c, fasting and post prandial glucose, and serum creatinine between the two groups. Serum concentrations of cytokines IL-6 (pg/mL) and TNF-α (pg/mL) were significantly higher ($p < 0.05$) in patients with than in those without PVD. In addition significant increase in WBC count ($p<0.05$) and neutrophil ($p<0.05$) percentage was observable in patient with PVD then patients without PVD. A significant increase in TC ($p<0.05$), TAGs ($p<0.05$), and LDL ($p < 0.05$) and concentrations was observed in PVD patients. HDL ($p<0.01$) was significantly lower in PVD patients. Furthermore ABI value was profoundly lower in PVD patients then those without PVD ($p<0.001$).

IL-6 was positively co-related with HDL with significance (r=0. 350 p= 0.028). TNF-α was only co-related with HbA1c with significant inverse pattern (r= -0.321, p= 0.045). ABI was negatively co-related with IL-6 (r= -0.347 p= 0.030) and TNF-α (r= -0.375, p= 0.018) with significance. There was no significant co-relation obtained between IL-6 and TNF-α with other variables (Table 2).

There was a positive and significant co-relation between ABI and age (β= 0.015, p= 0.018). Fasting and post prandial glucose along with duration of diabetes, BMI, HbA1c, TAC, TAGS, LDL, HDL were not significantly co-related with ABI. We found an inverse significant co-relation between ABI and systolic and diastolic blood pressures (β= -0.012, p= 0.016; β= -0.016, p=<0.0001) respectively. WBCs and neutrophils, markers of inflammatory response showed no significant co-relation with ABI (β= -0.460, p= 0.100) and (β= 0.004, p= 0.197) respectively. In all diabetic patients studied, ABI was negatively co-related with IL-6 concentration (β= -0.005 p= 0.030) (Fig 1A). ABI was also tended to negatively co-relate with TNF-α with a significance (β= -0.006, p=0.018) (Fig 1B) (Table 3).

To determine the independent influences of variables on ABI stepwise multiple linear regression analysis was performed. Age (β= 0.014, p= 0.003), HbA1c (β= -0.059, p= 0.014), systolic blood pressure (β= -0.012, p= 0.002), total cholesterol (β= -0.005, p= 0.015), IL-6 (β= -0.005, p= 0.003) and TNF-α (β= -0.005, p= 0.009) were independent predictors of ABI (Data not shown).

IV. CONCLUSIONS

The present study demonstrated significant negative co-relation between serum levels of IL-6 and TNF-α with ABI in patients with type 2 diabetes. A strong negative correlation of IL-6 and TNF-α with ABI suggest that chronic inflammation is important in atherosclerotic events leading to rise in pro-and anti-inflammatory cytokines. IL-6 plays a pivotal role in release of hepatic acute phase proteins such as C- reactive protein (CRP) and fibrinogen. An increase in intracellular adhesion molecule 1 (ICAMs) as a response to IL-6 levels causes homing of leukocytes is atheromatous plaques further transformation of smooth muscle cells (SMCs) into foam cells [12]. A destructive remodeling of the vessel structure with the formation of complex atherosclerotic lesions is preceded by release of IL-6 as a

consequence of prolonged inflammation [13, 14]. Thus evaluation of serum IL-6 concentration is a hallmark in diagnosing progression of atherogenesis. In this study inverse relationship clearly explains increased levels of IL-6 with decreasing ABI. TNF-α act as local intensification signal in pathological process associated with chronic inflammation in diseases like rheumatoid arthritis, myocardial infarction, and cardiovascular disease. In this study a significant negative co-relation between serum level of TNF-α and ABI portrays atherosclerosis as a low grade chronic inflammatory process. Previous study have reported TNF-α as a promising marker of progressive inflammation, and this study also supports TNF-α as capable predictor of lower extremity atherosclerosis. WBC was concluded as a single potential marker associated with the presence, severity and extent of coronary atherosclerosis [5]. Increased neutrophil count may reflect the burden of atherosclerotic induced tissue damage [15]. In this study leukocytes and neutrophils, established markers of inflammation were elevated with significant difference in PVD subjects suggesting their association with progressive vascular disease of foot. We found no significant difference in age between diabetic patients with and without PVD. Patients with PVD have longer onset of diabetes than without with a significant difference. This suggests the non-reversible effect of long standing diabetes making it a major risk factor of atherosclerotic vascular disease. BMI was significantly elevated in PVD patients. Previous studies have linked an increased BMI in peripheral arterial disease [16]. In both groups, those with PVD had significantly higher triglyceride, total cholesterol, LDL and lower HDL content than those without PVD. A prospective study demonstrated an altered lipid profile in patients with PVD [17].

In the present study we found a significant inverse co-relation of IL-6 and TNF-α with ABI suggesting the direct effect of pro-inflammatory cytokines in prognosis of atherosclerosis. This study strongly supports the hypothesis that increased IL-6 and TNF-α account for atherosclerotic events in lower extremities, however further investigations should me more deeply evaluated to reveal the underlying mechanism and elucidate the association of these inflammatory markers and vascular damage. Moreover, the characterization of beneficial and deleterious immune mediators in the process of atherogenesis in patients with diabetes would be important to identify potential therapeutic targets and immune-modulating treatment options. Our data also raise the possibility that inflammatory markers might provide an ancillary method for early disclosure of risk for PVD pathogenesis in type 2 diabetes. Combined use of inflammatory cytokines and ABI may have a greater predictive value of atherogenesis of lower extremities than either measure alone. Considering the importance of IL-6 and TNF-α in the development of peripheral arterial disease, targeting its actions could prove to be beneficial. This hypothesis warrants testing in a prospective study.

The present study is by far the first reporting the role of inflammatory markers in progressive atherogenesis in lower extremities among type 2 diabetic patients of northern Indian population. A replication of the present study is needed encompassing larger samples to explore the clinical relevance of these associations.

AUTHORS CONTRIBUTION

SN performed experimental work along with statistical calculations. JA and IP involved in the peer reviewing of the manuscript into final form and drafting of research idea of the present study.

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APPENDIX

Table 1: Demographic, clinical, and laboratory data for type 2 diabetes with or without PVD.

[1] Variables	[2] With PVD	[3] Without PVD
[4] N (M/F)	[5] 35 (25/10)	[6] 25(20/5)
[7] Age (years)	[8] 51.58±11.38	[9] 54.66±6.52
[10] BMI (kg/m^2)	[11] 31.12±11.79	[12] 23.54±4.09*
[13] Diab duration (yrs)	[14] 7.47±4.1	[15] 11±4.51*
[16] HbA1c (%)	[17] 8.14±1.96	[18] 8.59±1.96
[19] FPG (mg/dl)	[20] 174.6±60.99	[21] 190.9±90.3
[22] PPG (mg/dL)	[23] 215.75±83.64	[24] 244±106.4
[25] Sys BP (mm Hg)	[26] 130.33±13.78	[27] 121.2±10.89*
[28] Dia BP (mm Hg)	[29] 83±13.2	[30] 73.73±6.13*
[31] TC (mg/dL)	[32] 164.29±22.4	[33] 146.46±29.43*
[34] TAGs (mg/dL)	[35] 183.12±54.88	[36] 148.33±38.9*
[37] HDL (mg/dL)	[38] 39.4±6.66	[39] 47.62±10.11†
[40] LDL (mg/dL)	[41] 78±6.18	[42] 71.6±11.3*
[43] Se Cr (mg/dL)	[44] 1.31±0.475	[45] 1.22±0.71
[46] WBCs (x10^3/uL)	[47] 12.07±8.16	[48] 7.40±2.62*
[49] Neutrophils (%)	[50] 49.92±18.17	[51] 37.76±17.57*
[52] IL-6 (pg/mL)	[53] 177.74±23.87	[54] 159.5±28.45*
[55] TNF-α (pg/mL)	[56] 58.67±28.30	[57] 40±15.26*
[58] ABI	[59] 0.677±0.13	[60] 1.27±0.41‡

Data are means ± SD, or n (%), p* <0.05, p† <0.01, p‡ <0.001

Diab, diabetes; HbA1c, glycated haemoglobin; FPG, fasting plasma glucose; PPG, post prandial glucose; Sys BP, systolic blood pressure; Dia BP, diastolic blood pressure; TC, total cholesterol; TAG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; WBCs, white blood cells; TNF-α, Tumor necrosis factor alpha; IL-6, interleukin-6; ABI, ankle brachial index.

Table 2: Correlation of inflammatory markers with demographical and clinical variables and Ankle brachial index.

Variables	WBCs		Neutrophils		IL-6		TNF-α	
	r	p	r	p	r	p	r	p
Age (yrs)	-0.308	0.055	0.475	0.405	0.098	0.550	-0.086	0.602
BMI (kg/m^2)	0.236	0.148	-0.083	0.615	0.2458	0.131	0.218	0.181
Dia. duration (yrs)	-0.029	0.856	0.268	0.098	-0.230	0.158	0.157	0.339
HbA1c (%)	0.246	0.129	0.478	0.002	-0.134	0.416	-0.321	0.045*
FPG (mg/dl)	-0.024	0.881	0.128	0.434	-0.098	0.552	-0.07	0.665
PPG (mg/dl)	0.153	0.475	-0.129	0.545	-0.001	0.991	0.048	0.822
Sys BP (mm Hg)	0.234	0.150	-0.137	0.402	-0.032	0.842	0.139	0.397
Dias BP (mm Hg)	0.070	0.668	0.163	0.320	-0.134	0.416	0.085	0.606
TC (mg/dL)	0.029	0.856	0.007	0.964	0.205	0.210	0.058	0.723
TAGS (mg/dL)	0.041	0.800	0.041	0.802	0.001	0.999	0.154	0.348
HDL (mg/dL)	0.086	0.599	-0.183	0.264	0.350	0.028*	0.026	0.871
LDL (mg/dL)	0.166	0.310	-0.196	0.230	0.008	0.960	0.011	0.944
Se.Cr (mg/dL)	0.061	0.710	-0.062	0.705	0.089	0.589	0.147	0.369
WBCs (x10^3/uL)	NT		0.039	0.812	0.083	0.611	-0.057	0.730
Neutrophils (%)	0.039	0.812	NT		-0.083	0.615	-0.225	0.167
TNF-α (pg/mL)	-0.057	0.730	-0.225	0.167	0.104	0.525	NT	
IL-6 (pg/mL)	0.083	0.611	-0.083	0.615	NT		0.104	0.525
ABI	-0.267	0.100	0.210	.197	-0.347	0.030*	-0.375	0.018*

p* <0.05, p‡ <0.001

Diab, diabetes; HbA1c, glycated haemoglobin; FPG, fasting plasma glucose; PPG, post prandial glucose; Sys BP , systolic blood pressure; Dia BP; diastolic blood pressure; TC, total cholesterol; TAG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Sr. Cr, serum creatinine ;WBCs, white blood cells; TNF-α, Tumor necrosis factor alpha; IL-6, interleukin-6; ABI, ankle brachial index.

Table 3: Univariate analysis of relationships between ABI and characteristics of patients with type 2 diabetes.

Variables	ABI	
Age (yrs)	β	p
BMI kg/m^2)	0.015	0.018*
Diab Duration (yrs)	-0.009	0.148
HbA1c (%)	0.015	0.117
FPG (mg/dl)	-0.020	0.534
PPG (mg/dL)	0.001	0.960
Sys BP (mm Hg)	0.001	0.366

Dia BP (mm Hg)	-0.012	0.016*
TC (mg/dL)	-0.016	<0.0001‡
TAGs (mg/dL)	-0.004	0.114
HDL (mg/dL)	-0.001	0.221
LDL (mg/dL)	-0.011	0.079
Se. Cr (mg/dL)	-0.011	0.131
WBC (x10^3/uL)	-0.083	0.473
Neutrophils (%)	-0.460	0.100
TNF-α (pg/mL)	0.004	0.197
IL-6 (pg/mL)	-0.006	0.018*

β= linear regression coefficient

p* <0.05, p‡ <0.001

Diab, diabetes; HbA1c, glycated haemoglobin; FPG, fasting plasma glucose; PPG, post prandial glucose; Sys BP , systolic blood pressure; Dia BP; diastolic blood pressure; TC, total cholesterol; TAG, triglycerides; HDL, high density lipoprotein; LDL, low density lipoprotein; Sr. Cr, serum creatinine ;WBCs, white blood cells; TNF-α, Tumor necrosis factor alpha; IL-6, interleukin-6; ABI, ankle brachial index.

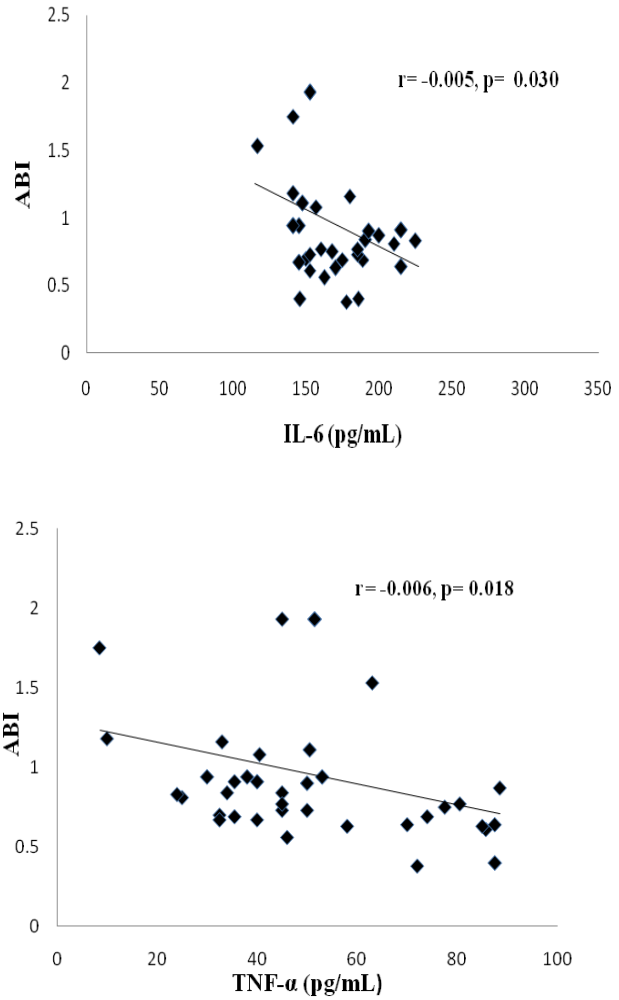

Fig 1: Correlation between serum concentrations of IL-6 (A) and TNF-α (B) with ABI.