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Vascular Inflammation Attributed by IL-6 and Thrombomodulin in Patients with Impaired Glucose Tolerance and Type 2 Diabetes Visiting a Local Hospital

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RS and SJ performed experimental work. TF presented the concept of the research. SS and RS collected the samples. SN, TF, SJ, FM and RS analysed the results. SN and SS wrote the manuscript. SJ, FM, SS and SN reviewed and approved the manuscript.

Key words
Atherosclerotic, Inflammation, Proatherogenic, Vascular

ABSTRACT

Diabetes mellitus type 2 (T2DM) is linked to a higher risk of atherosclerotic cardiovascular disease. The development and outcome of atherosclerosis are both influenced by vascular inflammation. The main purpose of this study is to find the impact of Interleukin-6 (IL-6) and thrombomodulin on vascular damage in the patients having impaired glucose tolerance (IGT) and to identify the relationship of IL-6 with insulin resistance. The patients visiting Amin Hayat Memorial Diabetic Center, Lahore from (January 2017 to October 2017) the subjects following WHO criteria of IGT and Diabetes were included. Demographic parameters as age, body mass index, waist/hip ratio, blood pressure, and socioeconomic status were recorded. Proatherogenic parameters like plasma glucose level, serum levels of insulin, thrombomodulin and IL-6 were measured by ELISA. The serum level of thrombomodulin was assessed as a measure of vascular damage. This study showed significantly elevated serum concentration of IL-6 and altered lipid profile in diabetes individual when compared with the control subjects. IL-6 and thrombomodulin was progressively higher in diabetic group. IL-6 correlated positively with thrombomodulin in the control group but an inverse association was seen in the IGT group and revealed that imbalance of the vascular damage initiated and that lead to damage of vascular endothelium in diabetes.

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INTRODUCTION

Diabetes mellitus is emerging as an epidemic all over the world. In a time of great stress, a higher prevalence is reported in women. It is present in stressful level in both developed and undeveloped countries (Zanardo *et al*., 2022). Pro-inflammatory cytokines and acute-phase markers are higher in impaired glucose tolerance (IGT) patients than in non-diabetic patients, according to cross-sectional research (Hu *et al*., 2022). Different cardiovascular diseases increase the level of C reactive proteins including Vonwillebrand factor and interleukin 6 (IL-6). It was assayed by measuring mCRP level in plasma. Atherosclerosis is a major cause of mortality is also a cardiovascular problem. Plaque formation increases the level of C reactive protein from baseline. Even at a site of small level of inflammation, plasma C reactive protein increase and bind to its ligand i.e., oxidation of LDL. Synthesis of proinflammatory cytokines (IL-6, IL-8) also increase and promote angiogenesis (Melnikov *et al*., 2022).

Advanced glycation end products (AGEs) are formed as a result of hyperglycemia at an accelerated rate which is an important biochemical abnormality in diabetic patients. Patients with T2DM have high levels of pro-inflammatory cytokines in their blood (Yamamoto and Sugimoto, 2016). IL-6 has been identified as an inflammatory cytokine that plays a significant role in the amplification of inflammatory reactions that cause atherosclerosis in many investigations (Akbari and Zadeh, 2018). IL-6 is an important cytokine that promotes atherosclerosis and increases the risk of cardiovascular complications 3 times. It is a multi-factorial cytokine that regulates immune responses, acute reactions, and hematopoiesis. IL-6 is an inflammatory cytokine that accelerates the formation of intercellular adhesion molecule-1 (ICAM-1). ICAM-1 is an important factor in the coagulation process and promotes coagulation

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reactions, thus enhancing the process of atherosclerosis (Emanuela *et al*., 2012). *In vitro* studies have shown that IL-6 has a pivotal role in insulin resistance acting directly on insulin resistance (Hartman and Frishman, 2014).

The levels of inflammatory cytokines TNF- and IL-6 have been shown to have a linear and positive connection with blood glucose levels. There is convincing evidence in the literature that T2DM causes alterations in inflammation and redox balance, as well as elevations in systemic markers such TNF- and IL-6 (Wei *et al*., 2022).

By delivering nitric oxide (NO) to the smooth muscles of the arteries, the endothelium helps to control the tone of the vasculature. It controls the amount of blood flow to various tissues based on their energy demands. The endothelium's malfunction has been linked to the onset and progression of atherosclerotic cardiovascular disease (CVD). In the natural history of type 2 diabetes, it has been demonstrated to occur before the beginning of hyperglycemia. IL-6 levels in the blood have been found to be higher in those with IGT or type 2 diabetes, and they have been linked to the development of T2DM. Thrombomodulin (TM) is a glycoprotein produced on the cell surface that is primarily generated by vascular endothelial cells and serves as an immediate cofactor for thrombin-mediated protein C activation (PC). The endothelial cell protein C receptor amplifies the phenomenon EPCR (Laishram *et al*., 2016).

Since there are few studies conducted on the association of IL-6 with vascular inflammation among IGT and type 2 diabetes patients (T2DM). This study aimed to find the impact of IL-6 on vascular damage in the patients having impaired glucose homeostasis. This research work will be a contribution to the new knowledge of inflammatory mediators' interaction with diabetes and atherosclerosis.

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MATERIALS AND METHODS

This case control study was conducted at Amin Hayat Memorial Diabetic Center, Lahore from January 2017 to October 2017. All cross sectional subjects were included in the study in this duration from diabetic center. A self-designed questionnaire, following WHO standards was designed to reveal information about socio-economic status, education, family history of diabetes, hypertension, smoking habits, and physical activity was filled. The study was carried out on 300 subjects with a range of age from 40–60 years. Sample size was calculated by raosoft software at 5% margin of error, 90% and expected population size of 20,000. 100 subjects were taken with IGT while 100 were selected as newly diagnosed with type-2 diabetes after oral glucose tolerance test. 100 age-matched healthy volunteers were selected as control group blood glucose less than 100mg/dl. Subjects were classified as having IGT with fasting glucose 101-125 mg/dl and the subjects having fasting glucose levels 126 mg/dl or above were classified as diabetic patients according to WHO standards. The subjects suffering from other disease like CVDs, neuropathy, kidney diseases and eye diseases were excluded from the study. The study protocol was approved by the Ethical Committee of Board of Directors at Amin Hayat Memorial Trust, Lahore.

Anthropometric characteristics such as height, weight, waist and hip circumference, were measured by standard measurement procedures of all participating subjects and blood pressure (mmHg) was also recorded by palpatory method. Then blood samples (6 ml) were drawn after a 12-h overnight fast via vacutainer by using BD syringes of 10cc with the help of professional technicians of the hospital. The blood samples were separated into two portions. One part of 3 ml was transferred to a red-capped vacutainer for serum preparation. Baseline levels of Insulin, IL-6, and TM in diabetic, IGT and control subjects were assessed by commercially available Calbiotech insulin ELISA kits.

The TM levels were measured as a marker of vascular endothelium injury and IL-6 as an inflammatory marker. The other portion of blood was transferred to purple capped vacutainer and was used for PBMC isolation and platelet count. Platelet count was determined by hematology analyzer, KX-21. PBMC isolation was performed for RNA extraction for studying gene expression profile of tumor necrosis factor (TNF). Lipid profile (total cholesterol, high-density lipoproteins (HDL), low-density lipoproteins (LDL) and triglycerides (TG) tests were also performed on chemistry analyzer (URIT-800) by using commercially available kits. STROBE case-control guidelines were used to report current study.

Statistical analysis

To present numerical data, Mean±SEM was employed. Differences in the means of the variables were analysed using both parametric and non-parametric tests, depending on the distribution of the variables. Prior to statistical analysis, the Kolmogorov-Smirnov test was employed to assess the normal distribution and homogeneity of variances.

All values were subjected to descriptive statistics, including age and BMI. In correlation (bivariate) analysis, Pearson's or Spearman's correlation coefficients with a 95 percent confidence interval were utilized. Analysis of variance was used to determine the difference between the groups (ANOVA). In a post hoc analysis, the Turkey test was used to detect the significant difference between the two groups.

To determine the difference between sexes, the independent sample students' test was performed to numeric data (Statistical Package for Windows, SPSS, Chicago, IL, U.S.A.). The standard error of the mean (SEM) calculated how much of a difference there is between a sample's mean and the population mean.

RESULTS

In this case control study, the enrolled 300 subjects were classified into three groups on the basis of blood sugar level i-e control (blood glucose <100mg/dl), IGT (101-125mg/dl) and diabetes bloodglucose (Table I). A significant positive relationship was found between IL-6 and BMI in all three groups. IL-6revealed a non-significant correlation with WHR in all three groups (Table II). Correlation between IL-6 and BSF was non-significant and negative in case of control and diabetic group while in IGT group it is non-significant and positive ($p>0.05$). The study revealed a significant correlation between IL-6 and total cholesterol in control and IGT group($p<0.05$) while diabetic group showed a non-significant, negative correlation($p>0.05$) (Table II).

The non-significant correlation was demonstrated between IL-6 and Triglycerides, the correlation was negative with IGT and diabetic group while it was positive in the control group in this parameter. A significant correlation was revealed between IL-6 and LDL in the control and IGT group, while the diabetic group showed a non- significant correlation. In IGT group the level of antithrombomodulin a bit increases with IL-6 cytokines (Fig. 1). A non-significant correlation was demonstrated between HDL and IL-6 in all the three groups ($p>0.05$), control and IGT group correlated negatively with IL-6. In diabetic patients the level of TM decreases with increase in IL-6 which shows antithrombin function. While the level of thrombomodulin remains more or less same in control group. It also cleared the relation between thrombomodulin function (antithrombin) and insulin resistance (Fig. 1).

IL-6 significantly correlated with LDL in the control and IGT groups. IL-6 showed an inverse correlation with HDL/LDL and positive correlation with total cholesterol/ HDL both in control and IGT group. Insulin and insulin resistance were high both in IGT and diabetic groups. IL-6 correlated significantly with insulin and insulin resistance only in the control group. Correlation between IL-6 and T. Cholesterol/HDL was positively significant in the control and IGTgroup. The diabetic group showed positive insignificant correlation. This study revealed a significant correlation of IL-6 with insulin only in the control group. IGT and diabetic group showed a non-significant correlation ($p>0.05$). According to this study a significant correlation was found between IL-6 and HOMA-IR only in the control group. IGT and diabetic group showed non-significant correlation ($p>0.05$).

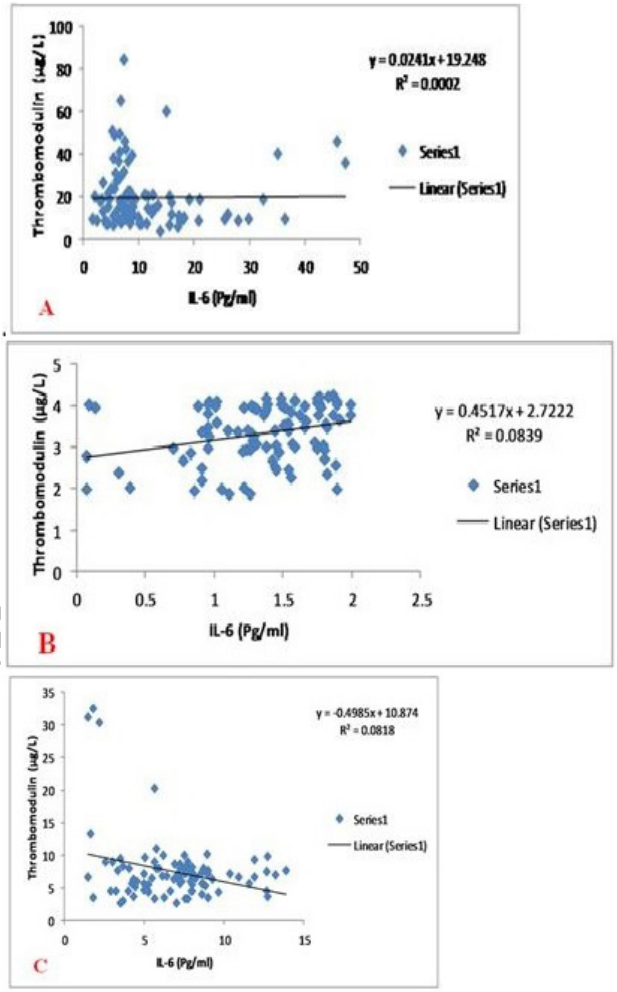

Fig. 1. Correlation analysis of IL-6 (pg/ml) with thrombomodulin (µg/L), in control (A), impaired glucose tolerance (B) and diabetic (C) group.

DISCUSSION

In our study, IL-6 had a significant difference in means among and between groups. The level of IL-6 was significantly higher in the IGT and diabetic group as compared to the control group (p and lt; 0.01). There was a 5-fold increase in the IGT group and an 8-fold increase in the diabetic group. The individual serum concentration of IL-6 varied considerably with the normal range covering a 9.6-fold difference in the IGT group and a 29-fold difference in the diabetic group. IL-6 was found to be strongly linked with BMI in all groups in this investigation

Table I. Demographic and biochemical characteristics of control, IGT and diabetic subjects (Mean ± SD).

Name of variable	Control (Range) (n=100)	IGT (Range) (n=99)	Diabetic (Range) (n=104)	p value
Age (years)	47.96±0.74 (40-60)	49.85±0.75 (40-60)	48.77±0.64 (40-60)	0.178
Height (cm)	162.52±1.15 (142-188)	156.74±1.13 (103-180)	158.31±0.92 (140-181)	0.001**
Weight (kg)	70.41±1.04 (46-89)	76.85±1.82 (42-158)	76.74±1.55 (44-130)	0.003**a,b
Body mass index (BMI)	26.77±0.39 (18.62-41.75)	31.19±0.77[1-2] (6.71-63.29)	30.69±0.59[1-3] (18.51-48.93)	0.002**
Waist (cm)	91.28±1.16 (56-113)	95.58±1.41 (63-140)	99.40±1.40[1-3] (55-135)	0.003**
Hip (cm)	105.34±1.19 (71-140)	108.53±1.41 (81-190)	110.41±1.32[1-3] (65-140)	0.029
WHR	0.86±0.01 (0.70-0.98)	0.88±0.01 (0.44-1.40)	0.89±0.01[1-3] (0.75-1.05)	0.004**
BSF (mg/dl)	82.66 ± 0.08 (62– 99)	111.15±0.66[2-3] (101-125)	183.17±4.43[1-3] (128-336)	0.001**
HbA1c (%)	5.07±0.03[1-2] (4.5-5.5)	5.99±0.02[2-3] (5.7-6.4)	9.77±0.23[1-3] (6.5-15.9)	<0.001**
Systolic BP (mm/Hg)	120.35±0.81 (100-130)	134.17±2.02[1-2] (100-180)	134.38±1.73[1-3] (110-190)	<0.001**
Diastolic BP (mm/Hg)	81.22±0.63 (70-95)	88.88±1.15[1-2] (80-140)	87.02±0.99[1-3] (70-120)	0.001**
T. Cholesterol (mg/dl)	174.96±1.20[1-2] (139-198)	191.21±3.61[2-3] (138-382)	214.78±4.45[1-3] (114-375)	< 0.001**
Triglycerides (mg/dl)	130.20±1.36 (92-150)	165.16±7.48[1-2] (65-503)	186.33±10.01[1- (70-807)	0.002**
LDL (mg/dl)	99.28±1.17[1-2] (71.8-123)	111.27±3.53[2-3] (65-311.2)	136.11±3.58[1-3] (62-221.4)	0.001**
HDL (mg/dl)	49.64±0.35 (43-62)	46.91±1.08[2-3] (30-62)	41.40±0.94[1-3] (25-72)	< 0.001**
LDL/HDL	2.02±0.02[1-2] (1.38-2.73)	2.43±0.09[2-3] (0.94-4.47)	3.42±0.11[1-3] (1.37-6.23)	< 0.001**
HDL/LDL	0.51±0.01 (0.37-0.73)	0.48±0.02[2-3] (0.22-1.07)	0.33±0.01[1-3] (0.57-0.16)	< 0.001**
T. Cholesterol/ HDL	3.54±0.03[1-2] (2.84-4.23)	4.27±0.15[2-3] (1.52-12.73)	5.40±0.15[1-3] (2.95-10.24)	< 0.001**
Insulin (µIU/ml)	6.57±0.27 (2.01-13.05)	17.44±1.55[1-2] (0.86-51.08)	18.29±2.40[1-3] (2.01-110.26)	< 0.001**
HOMA-IR	1.34±0.06[1-2] (0.38-3.10)	4.86±0.44[2-3] (0.23-15.26)	8.71±1.29[1-3] (0.76-89.02)	< 0.001**
IL-6 (Pg/ml)	1.36±0.04[1-2] (0.08-1.99)	6.88±0.29[2-3] (1.44-13.89)	10.99±0.84[1-3] (1.63-47.26)	< 0.001**
Thrombomodulin (µg/L)	3.34±0.07[1-2] (1.86-4.25)	7.44±0.51[2-3] (2.59-32.55)	19.51±1.39[1-3] (3.55-84.45)	< 0.001**

*, P<0.05 Significant difference between control and IGT; **, P<0.011. BP, blood pressure, WHR, waist-hip ratio; BSF, blood sugar fasling; HbA1c, hemoglobin; LDL, low density lipoprotein; HDL, high density lipoprotein; IL-6, interlenkin 6; HOMA-IR, homoostasis model assessment-estimated insulin resistance. [1-2], difference between control and IGT; [2-3], difference between IGT and diabetic; [1-3], difference between control and diabetic.

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Table II. Correlation analysis of IL-6 and Thrombomodulin with other parameters.

Variable	IL-6			Thrombomodulin		
	Control group	IGT	Diabetic	Control group	IGT	Diabetic
Body mass index (BMI)	0.508**	0.321**	0.477**	0.131	0.162	-0.122
Waist hip ratio (WHR)	0.110	0.087	0.070	0.163	-0.182	0.051
Blood sugar fasting (BSF)	-0.060	0.076	-0.044	-0.036	-0.073	0.411**
Systolic BP	0.345**	0.125	0.138	0.174	-0.011	0.255**
Diastolic BP	0.257*	-0.048	0.271**	0.259**	0.139	0.070
Total cholesterol	0.256*	0.201*	-0.007	0.017	-0.040	0.049
TG	0.011	-0.025	-0.045	0.180	0.129	0.173
Low density lipoprotein (LDL)	0.264**	0.262**	0.003	0.078	-0.169	0.004
High density lipoprotein (HDL)	0.009	-0.150	0.051	-0.060	0.074	-0.153
Insulin	0.462**	0.181	0.064	-0.185	-0.061	0.230*
Homeostasis model assessment-estimated insulin resistance (HOMA-IR)	0.433**	0.184	0.040	0.187	-0.060	0.326**
Platelet count	0.034	0.227*	-0.204	0.030	-0.124	0.074
Interleukin 6 (IL-6)				0.290**	-0.286**	0.015

*, P<0.05; **, P<0.01. For details of abbreviation, see Table I.

(p, 0.01). Another pro-inflammatory cytokine implicated in the development of atherosclerosis is IL-6. The non-enzymatic interaction between glucose and proteins or lipoproteins in artery walls, collectively known as the Maillard/browning reaction, is one of the key processes responsible for accelerated atherosclerosis in diabetics (Meo *et al*., 2016).

Early glycosylation products are formed when glucose reacts chemically reversibly with reactive amino groups of circulating or vessel wall proteins (Schiff bases), leading to the formation of more stable Amadori-type early glycosylation products (Ahmad *et al*., 2022). Our findings are in line with those of a study that found that IL-6 had a favourable relationship with BMI. Because adipose tissues release IL-6, there is a direct relationship between BMI and IL- 6 (Kreiner *et al*., 2022). Individuals become fat as their adipocytes become larger and their adipose tissue undergoes molecular and cellular changes that disrupt systemic metabolism. Firstly, macrophage numbers increase in adipose tissue with obesity (Klein *et al*., 2022). They apparently function to search older adipocytes. Second, as obesity increases, various pro-inflammatory substances are generated in adipose tissue macrophages. Almost all adipose tissue tumour necrosis factor (TNF-) expression and large levels of interleukin-6 are produced by macrophages (IL-6) (Huang *et al*., 2022).

The cellular responses to IL-6 via trans signaling versus conventional signaling may impact the effect of IL-6 on endothelial cells. People with T2DM have higher levels of acute-phase response markers and inflammatory mediators such cytokines like interleukin-6. *In vitro* endothelial cell capillary-like network formation is reduced by circulating components found in the serum of T2DM patients, and this is consistent across primary and telomerized cell lines. The deficits in angiogenic activities are mostly due to the increases in IL-6 and sIL-6R observed in T2DM. This discovery has led to the hypothesis that elevated levels of pro-inflammatory cytokines and the resulting acute-phase response may be at the root of much of the metabolic clustering associated with obesity and diabetes (Yalçın *et al*., 2022).

Our results show a relationship between IL-6 and diastolic blood pressure in the diabetic group. According to one report, apparently healthy subjects, IL-6 is an independent risk factor for high blood pressure (Elsayed *et al*., 2022). Recently a study is conducted on the link of IL-6 with hypertension in diabetic subjects. They reported that hypertension in type 2 diabetes with high BMI is dependent on the rise of inflammatory marker IL-6 (Boarescu *et al*., 2022). Many previous studies have reported that IL-6 levels become high in IGT and diabetic subjects and elevated levels of IL-6 are linked with higher blood pressure (Hossain *et al*., 2022).

In the T2DM acute phase, responses have been reported. According to one study IL-6 is an acute phase marker that is increased in T2DM. The production of acute-phase proteins is stimulated by IL-6 in the liver. The impact of IL-6 on the brain, which stimulates the release of adrenocorticotropic hormone (ACTH) and growth hormone, is one probable mechanism for the

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creation of insulin resistance. Another probable reason for the formation of insulin resistance is the inhibition of pancreatic cells (Tyfoxylou *et al*., 2022).

TM is a glycoprotein present on the surface of endothelial cells. It can bind to thrombin and activate protein C. In this way it can reduce the effects of cytokines in inflammatory and immunological processes. TM is traditionally thought to serve as a marker of endothelial cell damage. The increase in serum concentration of soluble TM may as results in a reduction of the membrane concentration level of TM as the endothelial membrane TM is directly responsible for the production of plasma TM (Takeshi, 2022). In our study, the level of TM is significantly higher in subjects with IGT and diabetes.

CONCLUSION

The substantial relationship between IL-6, lipid profile, TM, and blood pressure in IGT and T2DM people reveals a network of pathophysiological alterations in the vasculature. Because TM is a glycoprotein found on the surface of endothelial cells, it exhibited a strong association with insulin and insulin resistance in diabetics. Protein C is activated when it binds to thrombin. As a result, the impact of cytokines in inflammatory and immunological processes can be reduced. As a result, TM has both a protective and antithrombotic effect.

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Ethical statement

The research study was approved ethically by Letter No. Zoo/LCWU/819, dated 05-02-2017. Blood sampling and further analysis was strictly in accordance with the guidelines provided by World Medical Association (WMA), declaration (2000) regarding personal equipment, disposal of wastes and storage of sample etc.

Statement of conflict of interest

The authors have declared no conflict of interest.

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