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To study vascular cell adhesion molecule-1 and remnant lipoprotein cholesterol in diabetes mellitus without retinopathy

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Abstract
Background: The aim was to look into the role of vascular cell adhesion molecule-1 and remnant lipoprotein cholesterol in patients of type 2 diabetes without retinopathy and its comparisons with the healthy non-diabetic controls.
Material and methods: 35 normotensive newly diagnosed Type 2 diabetic patients with retinopathy and 15 healthy normotensive nondiabetic age and sex match controls selected. Cases and controls were taken from 1st June 2017 to 31st June 2018. All cases undergoes thorough investigation. The obtained information regarding the levels of fasting blood sugar (FBS), post prandial blood sugar (PPBS), Glycosylated Hemoglobin level (Hb1Ac), Lipid Profile, 24 hours urinary protein, VCAM-1 Levels, Remnant lipoprotein levels were analyzed, the statistical analysis was performed using SPSS for windows version 16.0 software. For comparing two group of mean Students't' test and for paired samples Paired 't' test was used. The p value <0.05 was considered as statistically significant.
Results: In our study majority of the patients were in age between 51 and 60 years. Male outnumbered females with ratio of 1.8:1. The mean level of FBS, PPBS, HbA1c, 24 hours urinary protein, triglyceride, VLDL, and VCAM were significantly higher in patients than in controls, in the meantime the mean value of HDL were significantly higher in controls than the patients. The positive correlation was seen in between VCAM with both HbA1c and 24 hrs. Urinary protein.
Conclusion: we have shown that soluble VCAM-1 may be a marker of chronic hyperglycemia and may be related to development of diabetic retinopathy and nephropathy. It may reflect systemic endothelial dysfunction. Remnant cholesterol is an important inflammatory marker leading to atherogenicity and amplifies further risk of micro and macrovascular complication. The data is scare for both marker in our region and need further study with large population size.

Keywords: Vascular cell adhesion molecule, remnant lipoprotein cholesterol, diabetes mellitus, retinopathy

Introduction
Diabetes mellitus (DM) is one of the most common non-communicable diseases globally. According to the number of patients, India (69.2 millions) ranks second in the world, and China (109.6 millions) ranks first in 2015 [1]. Diabetes is usually associated with the increased production of inflammatory mediators [2].
Diabetic Retinopathy is currently estimated to be the leading cause of new onset blindness in working-aged adults in developed countries [3]. Based on clinical observations, it was initially assumed that the microvascular complications only began to develop into the early years of natural history of DM. A number of interconnecting biochemical pathways have been proposed as potential links between hyperglycemia and diabetic retinopathy. These include increased polyol pathway flux, activation of diacylglycerol-(DAG) PKC pathway, increased expression of growth factors such as vascular endothelial growth factor (VEGF) and insulin-like growth factor-1 (IGF-1), hemodynamic changes, accelerated formation of advanced glycation end products (AGEs), oxidative stress, activation of the renin-angiotensin-aldosterone system (RAAS), and subclinical inflammation and leukostasis [4].
Increasing evidences in both the experimental and clinical studies suggest that oxidative stress plays a major role in the pathogenesis of diabetes mellitus [5]. Leukocyte adhesion to the vascular endothelium is one of the necessary first steps in atherogenesis, as occurs in Diabetic Retinopathy. These interactions are mediated by membrane adhesion molecules expressed on leukocytes such as leukocyte function antigen-1(LFA-1) and very late antigen-4(VLA-4) and

their respective vascular endothelial ligands such as vascular cell adhesion molecule-1 (VCAM-1) [6].

Elevated RLP cholesterol (RLP-C) levels were reported to be associated with endothelial dysfunction, an early marker for atherosclerotic disease [7]. The Framingham Heart Study found that an increase in RLP-C levels was a significant risk factor for coronary artery disease in women [8]. According to Varbo *et al.* (2013) higher level of fasting remnant lipoprotein cholesterol is associated with higher level of serum CRP level. However its contribution to development of diabetic retinopathy remains unexplored.

The data pertaining to the systemic inflammation and dyslipidemia and serum vascular cell adhesion molecule-1 and remnant lipoprotein cholesterol in the type 2 diabetics without retinopathy are scanty from our country and especially from this region. Present study is aimed to look into the role of vascular cell adhesion molecule -1 and remnant lipoprotein cholesterol in patients of type 2 diabetes without retinopathy and its comparisons with the healthy non-diabetic controls.

Material and methods

The present study was conducted in the Department of General Medicine, Institute of Medical Sciences, Banaras Hindu University Varanasi, in collaboration with Department of Biochemistry in the period of month of June 2017 to July 2018.

35 patients of newly diagnosed type 2 diabetics without retinopathy of age between 20 to 65 years were selected from the Department of Medicine and Endocrinology IMS, BHU, Varanasi. 15 Age and sex matched healthy non-diabetic & Normotensive individuals were selected as the controls.

Detailed history and clinical examination (including fundoscopy by ophthalmologist) was done in all the selected cases and controls. Then the venous blood samples of about 5 ml were collected. 3 ml was taken in a clean and dry plain vials without any anticoagulant. The blood was allowed to clot at room temperature. The sera was removed and stored at -20^{0}C in a sterile plain glass vial until analyzed. 2ml of blood was also taken in EDTA vial for analysis.

Before final estimation of VCAM and remnant lipoprotein cholesterol level all the cases and controls underwent complete blood counts, Renal function test, Liver function test, Plasma glucose fasting and post prandial, HbA1c, Urine R/M, 24 hour urinary protein, Electrocardiogram, 2D echocardiogram (optional, clinically suspected coronary artery disease patients only).The Criteria for diagnosis of diabetes was adapted from American Diabetes Association 2017. The patient were excluded who were on multi vitamins and mineral therapy, smoking, known diabetic, who were working in chemical / asbestos / metal factories, receiving chemotherapy and radiotherapy, with hypertension, on drugs like metformin, linagliptin and glibenclamide.

Estimation of remnant lipoprotein cholesterol

Remnant lipoprotein cholesterol level was estimated with the use ELISA method (Cat. No: MBS044939). Using Purified Human RLP-C antibody to coat Microelisa Stripplate wells to make solid-phase antibody, then add RLP-C and RLP-C antibody which has been labeled with HRP to wells, then the reactants become antibody-antigen-antibody-enzyme complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue color under HRP enzyme-catalyzed, reaction is terminated by the addition of a sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450 nm. The concentration of RLP-C in the samples is then determined by comparing the O.D. of the samples to the standard curve. The sensitivity of this kit is 5.0µmol/L. The detection range of this kit is 31.2µmol/L-1000µmol/L. No significant cross-reactivity or interference between Human RLP-C and analogues was observed. Both Intra-assay CV (%) and Inter-assay CV (%) is less than 15%. [CV (%) = SD/mean ×100]. The loss activity rate of this kit is less than 5% within the expiration date under appropriate storage condition. The results were calculated by averaging the duplicate readings for each standard and sample to subtract average optical density of the Blank/Control (VB/C), using the professional curve fitting software to make a standard curve and calculate the concentration of the samples.

Estimation of vcam-1 level

VACM-1 level was estimated with the use of Ray Bio® Human VCAM-1 ELISA Kit (Catalog #: ELH-VCAM-1). This kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human VCAM-1 in serum, plasma, and cell culture supernatants. This assay employs an antibody specific for human VCAM-1 coated on a 96well plate. Standards and samples are pipetted into the wells and VCAM-1 present in a sample is bound to the wells by the immobilized antibody. The wells are washed and biotinylated anti-human VCAM-1 antibody is added. After washing away unbound biotinylated antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of VCAM-1 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm. The entire kit may be stored at -20°C for up to 1 year from the date of shipment. Avoid repeated freeze-thaw cycles. The kit may be stored at 4°C for up to 6 months. For extended storage, it is recommended to store at -80°C.

The calculation of the mean absorbance for each set of duplicate standards, controls and samples, and subtract the average zero standard optical density need to be done. Plot the standard curve on log-log graph paper or using Sigma plot software, with standard concentration on the x-axis and absorbance on the y-axis. Draw the best-fit straight line through the standard points. The minimum detectable dose of Human VCAM-1 was determined to be 300 pg/ml. Minimum detectable dose is defined as the analyte concentration resulting in an absorbance that is 2 standard deviations higher than that of the blank (diluent buffer). This ELISA kit shows no cross-reactivity with any of the cytokines tested.

Statistical analysis

The statistical analysis was done using SPSS for Windows version 16.0 software. Descriptive statistics like mean, frequency and percentages of various parameters were calculated. For categorical variable Chi-Square test and Fischer's Exact test was used. For comparing two group of mean Students't' test and for paired samples Paired 't' test was used. The p value <0.05 was considered as statistically significant.

Observation and results

The present study was conducted in the Departments of Medicine and Department of Biochemistry of Institute of

Medical Sciences, Banaras Hindu University. The study consisted of 34 patients of type 2 Diabetics without retinopathy and 15 healthy non-diabetic and non-hypertensive age and sex matched controls.

In our study a total of 49 patients were selected, of which 29 (59.2%) were male and 20 were female (40.8%).The male: female ratio was 21:13 in cases and 8:7 in control group. There were 61.8% male in the case group and 53.3% in the control group (Table 1). In the patient group mean age was 50.79±9.37 and in the healthy control group mean age was 55.27±5.78 (p value =0.095) (Table 3). In the patient group 20.6% 7) patients were in the age group of 31-40, none in the control group. 20.6% 7) patients were in the age group of 41-50, while 26.7% (4) in the control group. 50.0 % 17) were in the age group of 51-60 in the patient group 53.3% 8) in the control group. 8.8% patient were > 60 in the patient group and 20% in the control group (Table 4).

In the present study fasting blood sugar was significantly elevated in the diabetic patients with retinopathy(mean 216.21 ± 72.75 mg/dl) than in the control group(88.33 ± 13.69 mg/dl) p <0.001 (Table 5). In present study post prandial blood sugar was significantly high in the diabetic patients with retinopathy (mean 307.76 ± 97.04 mg/dl) than in the control group(124.20 ± 9.28 mg/dl) p <0.001 (Table 6) HBA1c was significantly high in the diabetic patients with retinopathy (mean 9.57 ± 1.83) than in the non-diabetic and non-hypertensive controls (mean 5.60 ± 0.50) p <0.001 (Table 7) In the present study, 24 hours urinary protein was significantly elevated in the patient group as compared to healthy control group. The mean level in the patient group was 66.85 ± 56.55 and mean level in the control group was 30.93 ± 18.44. (p value =0.011) (Table 8) There was no significant difference in the serum cholesterol between the two groups.

Mean serum cholesterol level in the patient group was 191.56±30.47 and the mean level in the healthy control group was 180.67±12.97. (P value > 0.05) (Table 9). In the present study, the mean serum triglyceride level in the patient group was 210.79±17.38. In the healthy control group, the mean triglyceride level was 134.33±8.43. The difference was significant with p=value < 0.001. (Table 10). In the present study, the mean serum VLDL level was 41.09±4.47 in the patient group and 21.93±4.38 in the healthy control group. The difference was significant. (Table 11). In our study, the mean serum LDL cholesterol level was 101.97±19.79 in the patient group and 92.27±10.72 in the healthy control group. The difference was insignificant. (P value > 0.05) (Table 12).

In the present study, the mean serum HDL cholesterol level was 33.68±7.16 in the patient group and 43.80±5.28 in the healthy control group. The difference was highly significant. (P value <0.001) (Table 13). In the present study, the mean concentration of VCAM-1 was 46.66±10.65 ng/ml in the diabetic patients and 25.35±6.66 ng/ml in the healthy control group. The difference was highly significant. (P value<0.001) (Table 14). In the present study the mean concentration of remnant lipoprotein cholesterol was 219.27±110.83 micro mol /liter in the patient group and 204.34±59.13 micro mol /liter in the healthy control group. The difference was insignificant. (P value> 0.05) (Table 15)

Table 1: Clinical characteristics of study Population

Parameter	Group I (n=34)	Group II (n=15)
Age (in years)	50.79±9.37	55.27±5.78
Male / Female	21 : 13	8 : 7

Table 2: Gender distribution

Sex	Group I (n=34)	Group II (n=15)	Total (n=49)
Male	21 (61.8%)	8 (53.3%)	29 (59.2%)
Female	13 (38.2%)	7 (46.7%)	20 (40.8%)
Total	34 (100.0%)	15 (100.0%)	49 (100.0%)

Table 3: Age group of study population

Group	Age (Mean ±SD)	t-value	p-value
Group I (n=34)	50.79±9.37	-1.705	0.095
Group II (n=15)	55.27±5.78		

Table 4: Distribution of age in study population

Age group	Group I (n=34)	Group II (n=15)	Total (n=49)
31-40	7 (20.6%)	0 (0.0%)	7 (14.3%)
41-50	7 (20.6%)	4 (26.7%)	11 (22.4%)
51-60	17 (50.0%)	8 (53.3%)	25 (51.0%)
>60	3 (8.8%)	3 (20.0%)	6 (12.2%)
Total	34 (100.0%)	15 (100.0%)	49 (100.0%)

c2=4.344, p=0.227

Table 5: Fasting Blood Sugar in the study population

Group	FBS (Mean ±SD)	t-value	p-value
Group I (n=34)	216.21 ± 72.75	t=6.717	p<0.001 (HS)
Group II (n=15)	88.33 ± 13.69		

Table 6: Post Prandial Blood sugar in the study population

Group	PPBS Mean ±SD)	t-value	p-value
Group I (n=34)	307.76 ± 97.04	t=7.269	p<0.001 (HS)
Group II (n=15)	124.20 ± 9.28		

Table 7: HbA1C % in the study population

Group	HbA1c Mean ±SD)	t-value	p-value
Group I (n=34)	9.57 ± 1.83	t=8.199	p<0.001 (HS)
Group II (n=15)	5.60 ± 0.50		

Table 8: 24 hrs. urinary protein in the study population

Group	24 hrs. urinary protein mg/dl) (Mean ±SD)	Mann Whitney U-test	P value
Group I (n=34)	66.85 ± 56.55	Z = -2.539	0.011 (S)
Group II (n=15)	30.93 ± 18.44		

Table 9: Serum Total Cholesterol level in the study population

Group	Cholesterol (Mean ±SD)	t-value	p-value
Group I (n=34)	191.56 ±30.47	t=1.326	p>0.05 (NS)
Group II (n=15)	180.67±12.97		

Table 10: Serum Triglyceride level in the study population

Group	Triglyceride (Mean ±SD)	t-value	p-value
Group I (n=34)	210.79±17.38	t=16.150	p<0.001 (HS)
Group II (n=15)	134.33±8.43		

Table 11: Serum VLDL Cholesterol in the study population

Group	VLDL (Mean ±SD)	t-value	p-value
Group I (n=34)	41.09±4.47	t=13.895	p<0.001 (HS)
Group II (n=15)	21.93±4.38		

Table 12: Serum LDL Cholesterol in the study population

Group	LDL (Mean ±SD)	t-value	p-value
Group I (n=34)	101.97±19.79	t=1.780	p>0.05 (NS)
Group II (n=15)	92.27±10.72		

Table 13: Serum HDL Cholesterol level in the study population

Group	HDL (Mean ±SD)	t-value	p-value
Group I (n=34)	33.68±7.16	t=-4.905	*P*<0.001 (HS)
Group II (n=15)	43.80±5.28		

Table 14: VCAM-1 in the study population

Group	VCAM 1(ng/ml) (Mean ±SD)	t-value	p-value
Group I (n=34)	46.66±10.65	t=7.131	*P*<0.001 (HS)
Group II (n=15)	25.35±6.66		

Table 15: Remnant Lipoprotein-cholesterol in the study population

Group	Remnant-Lipoprotein cholesterol (micro mol/liter)	t-value	p-value
Group I (n=34)	219.27±110.83	t=0.490	*P*>0.05 (NS)
Group II (n=15)	204.34±59.13		

We have found statistically significant positive correlation between HbA1C % and level of soluble VCAM-1. Pearson correlation = 1.7324, p value <0.05)

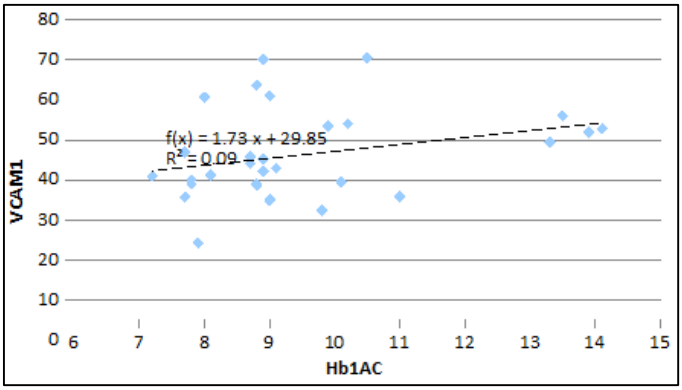

Fig 1: Correlation between Hb1AC versus VCAM 1

Our study has shown statistically significant positive correlation between VCAM-1 and 24 hours urinary protein. (Pearson correlation = 0.0485, p value < 0.05)

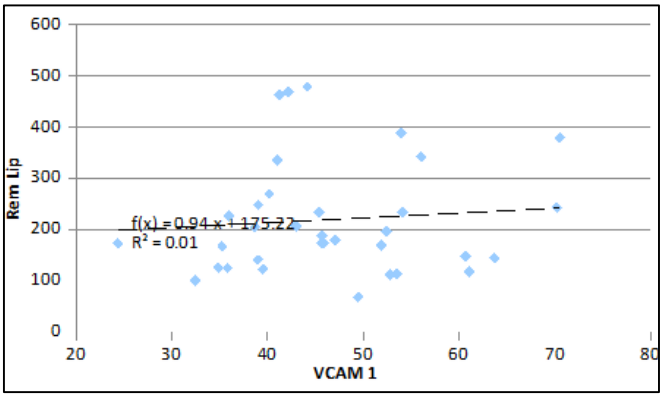

Fig 2: Correlation between VCAM -1 and 24 hours urinary protein

Discussion

The present study involved 34 patients of type 2 Diabetes mellitus without retinopathy, and 15 healthy normotensive non-diabetic age and sex matched healthy controls. The objective of the study was to compare the levels of vascular cell adhesion molecule-1 and remnant lipoprotein cholesterol in the above subjects.

Diabetes mellitus is a metabolic disorder associated with macrovascular (coronary artery, cerebrovascular and peripheral vascular disease) and microvascular (retinopathy, nephropathy, and neuropathy) complications. Diabetes is associated with the increased production of the free radicals and impaired antioxidant defenses [9].

Vascular cell adhesion molecule-1

M. Koga *et al* (1998) evaluated in 95 Japanese patients with Type 2 diabetes mellitus (DM) the level of VCAM-1. Serum soluble VCAM-1 concentration was higher in patients with more advanced stages of retinopathy as well as nephropathy. There was a significant correlation between soluble VCAM-1 and urinary albumin excretion in 69 patients with normal serum creatinine levels [10] ($r = 0.51$, $p < 0.0001$). Soluble VCAM-1 concentration increases with progression of nephropathy, compatible with reports by Schmidt *et al* that soluble VCAM-1 concentration was elevated in diabetic patients with microalbuminuria. Matsumoto *et al* (2002) have studied the correlation between serum concentrations of soluble adhesion molecules and diabetic microangiopathy in 26 patients with type 2 diabetes. The found that the VCAM-1 level was significantly higher in the microangiopathy group than in the control group [11].

In the present study we have demonstrated that level of VCAM-1 molecule is significantly elevated in diabetic patients in comparison to healthy controls (p value < 0.001 HS), consistent with Matsumoto *et al* . In our study, the level of soluble VCAM-1 correlated significantly with the 24 hours urinary protein excretion, compatible with reports by Matsumoto *et al* that soluble VCAM-1 concentration was elevated in diabetic patients with microalbuminuria.

The level of soluble VCAM-1 also correlated significantly with HbA1C % p value < 0.05). This may reflect the effect of chronic hyperglycemia on endothelial dysfunction consistent with the findings of Morigi *et al.*

In our study we compared type-2 diabetes mellitus patient without retinopathy and healthy subjects not having any disease. The mean value of VCAM-1 in diabetic patient without retinopathy was 46.66±10.65 and mean value in healthy subjects was 25.35±6.66. The P value of study was *P*<0.001 which was highly significant. So, there is positive correlation between VCAM -1 level in type-2 diabetic patient without retinopathy.

Remnant lipoprotein cholesterol

According to Goliasch, high remnant cholesterol is more predictive of myocardial infarction than any other lipid particle. Remnant cholesterol is especially predictive of coronary artery disease in patients with normal total cholesterol [12]. Bernelot Moens SJ *et al.* have demonstrated inflammatory component to atherogenesis of remnant lipoprotein cholesterol, contributing to cardiovascular disease risk in patients with familial dysbetalipoproteinemia [13]. However its contribution to development of diabetic microvascular complications remains unexplored.

The present study revealed highly significant relations between Triglycerides, HDL and VLDL molecules in Patient of diabetes without retinopathy and controls. The value of HDL is significantly higher in controls as compared to cases. The present study did not show any statistically significant difference in the concentration of remnant lipoprotein cholesterol between patients with diabetes without retinopathy and healthy controls. There was no significant correlation between the level of VCAM-1 and remnant lipoprotein cholesterol.

Conclusion

We have not found any statistically significant difference in the level of remnant lipoprotein cholesterol between diabetes patients without retinopathy and healthy controls. But we found positive correlation between HbA1c and 24hr urinary protein with VCAM-1 level. Thus in conclusion, we have shown that soluble VCAM-1 may be a marker of chronic hyperglycemia and may be related to development of diabetic retinopathy and nephropathy. It may reflect systemic endothelial dysfunction.

The above findings imply an important inflammatory component to the atherogenicity of remnant cholesterol, contributing to the increased micro and macrovascular risk of diabetes mellitus. The role of remnant lipoprotein cholesterol in pathogenesis of diabetes mellitus and development of its complications needs further evaluation with larger sample size.

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