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Lecithin: Cholesterol Acyltransferase Activity and Homocysteine Levels are Better Associated with Lipid Profile Indices Compared to Glycemic Control Indices in Patients with Diabetes Mellitus

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Authors' contributions

This work was carried out in collaboration between all authors. Author MN designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author ZG designed the study, analyzed data and wrote manuscript. Author ZB managed the literature searches, collected data and wrote the first draft of the manuscript. Author NA analyzed data and wrote the first draft of the manuscript. Authors AAN and AM analyzed data and collected data. Authors AG, AE and HM managed experimental process. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Homocysteine (Hcy) level, LCAT activity, and HDL-C concentration and oxidative/anti-oxidative capacities are well-established factors in determining cardiovascular disease risk in Type 2 diabetes mellitus. However, the association between these factors and lipid profile and glycemic control variables remains to be unveiled. We aimed to assess accuracy of LCAT activity and Hcy

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for dyslipidemia and poor glycemic control screening in patients with type 2 diabetes mellitus.
Study Design: This case control study was conducted in Vali-Asr hospital affiliated with Tehran University of Medical Sciences.
Methodology: We quantified FBS, HDL-C, HbA1c, Hcy, LCAT activity and other lipid profiles markersin a total of 41 patients with type 2 diabetes mellitus along with 41 matched healthy subjects.
Results: Linear regression and correlation analysis revealed that Hcy had a positive correlation with LCAT activity only in HDL levels above 40 mg/dl. Receiver operating characteristics (ROC) curves testing LCAT activity in prediction of hyperhomocysteinemia as the state variable for both control subjects and patients, showed the one for patients with diabetes being more shifted up and to the left than for control subjects. In addition, both Hcy and LCAT activity predicted lipid profile variables more precisely than glycemic control variables.
Conclusion: LCAT activity could be considered as one of the factors predicting hyperhomocysteinemia and therefore indirectly associated with cardiovascular events and it is more specific and sensitive in patients with diabetes compared to control subjects. However neither Hcy and nor LCAT activity, are able to predict diabetes and/or diabetes associated oxidative stress. These factors might be more associated with diabetes related dyslipidemia.

Keywords: Homocysteine; lecithin: cholesterol acyltransferase; high-density lipoprotein, type 2 diabetes mellitus.

1. INTRODUCTION

Type 2 diabetes mellitus (T2DM) is associated with a sharply increased risk of cardiovascular disease [1-3]. Increased total plasma homocysteine (Hcy) level and serum lipidshas been claimed to be a risk factorfor cardiovascular diseaseand are clinical parameters that are commonly evaluated in determining cardiovascular [4] and other complications risks in patients with T2DM [5-7]. Evidence for this has come frommeta-analysis based on several epidemiological studies and also from prospective studies [8,9]. However, the results of randomized trials with homocysteine lowering treatment have failed to show that homocysteine is a causal risk factor [10,11]. Thereafter, numerous studies began to investigate for possible mechanisms that Hcy may contribute to cardiovascular disease, such as inflammation, oxidative stress and enhanced lipogenesis [12]. Various studies have been performed to highlight factors influencing Hcy level and alterations. Some studies reported a negative correlation between homocysteine and High-DensityLipoprotein (HDL) cholesterolin patients with coronary heart diseases [13,14].

HDL removal from tissues depends on the activity of lecithin: cholesterol acyltransferase (LCAT), a soluble enzyme that convert cholesterol to cholesteryl ester on the surface of HDL [15]. Thus HDL is a chief site for esterification of cholesterol by LCAT and LCAT activity could be an indicator of serum cholesterol clearance [16]. Studies identified that LCAT activity decreases in T2DM via hyperglycemia-induced glycation of HDL and LCAT [17,18]. Moreover, LCAT activity is correlated negatively with HDL'santi-oxidative characteristics and therefore it can be considered as an indirect indicator of HDL's anti-oxidative abilities [19,20].

Here we report: 1) the relationship of plasma HDL-C, LCAT activity, Hcy and other serum biochemical variables 2) Hcy and LCAT activity accuracy in predicting lipid profile and glycemic control related variables, states associated with increased cardiovascular events, in patients with type 2 diabetes mellitus.

2. MATERIALS AND METHODS

2.1 Setting and Participants

This case control study was conducted in Vali-Asr hospital affiliated with Tehran University of Medical Sciences. A total of 41 patients with type 2 diabetes mellitus who attended the outpatient diabetes clinic of the hospital, along with 41 healthy persons who matched in age, sex and Body mass index (BMI) with patients, were enrolled in the study. The research was performed according to the Declaration of Helsinki principles and was approved by the ethics committee of the Tehran University of Medical Sciences.

All subjects underwent physical examination and questionnaires were completed including age, height, weight, medication, medical history,

smoking habits, and a family history of coronary heart disease, blood pressure and diabetes.Diabetes was diagnosed according to the criteria of American Diabetes Association (fasting plasma glucose ≥126 mg/dl (7.0 mmol/l)). BMI was calculated as the ratio of weight (kg) to the square of height (m^2). Blood pressure (systolic and diastolic) was measured in the sitting position after 10 minutes rest and the average of 2 measurements with 5 minutes interval. Hypertension was defined as systolic BP >140 mmHg and /or diastolic BP> 90 mmHg, or current use of antihypertensive medications.

None of the participants had a major clinical problem and none of them were taking aspirin, metformin, insulin, lipid lowering drugs and antioxidant, vitamin supplementation and drugs influencing plasma total homocysteine level such as methotrexate, phenytoin, fibrates, oral contraceptives, sulfonamides.

2.2 Laboratory Measurements

Blood samples were collected following an overnight fasting (between 10-12 hours) and then were transferred into EDTA-containing tubes (1.5 mg / ml) for biochemical and other study parameters measurement. Plasma was obtained by centrifuging blood samples at 3000 rpm for 15 min at 4°C. Serum lipids and other metabolites were measured shortly after blood collection using standard procedures.

Samples for measurement of homocysteine and LCAT activity were stored at -70°C until analysis. Fasting blood glucose, total cholesterol, triglycerides, were measured using enzymatic methods (Pars Azmoon, Iran). HDL-C, LDL were assessed by direct enzymatic colorimetric assays (Pars Azmoon, Iran). Plasma creatinine measured by using jaffe reaction (Pars Azmoon, Iran).Glycated hemoglobin (HbA1c) was determined by high-performance liquid chromatography (HPLC).

Plasma lecithin cholesterol acyltransferase (LCAT) activity level was measured by fluorometric assay kit (Calbiochem, Germany). Briefly, human plasma samples were incubated with LCAT substrates. Hydrolyzing the substrate by LCAT, results in a product with emission at 390 nm wavelengths. However, the intact substrate has a maximum fluorescence emission at 470 nm wavelength. The LCAT activity was assessed as a change in 470 / 390 emission intensity.

Fasting total homocysteine (Hcy) plasma concentration was determined by high-performance liquid chromatography (HPLC) with fluorescence detection(Shimadzu RF-10AXL Fluorescence Detector, Germany), involving a fluorogenic reagent, ammonium 7-fluorobenzo-2-oxa-1, 3-diazole-4-sulphonate (SBD-F) and included all molecular forms that can be reduced to free Hcy.

2.3 Statistical Analysis

The data were analyzed using statistical software package software (SPSS), version 17.0 (Chicago, Illinois, USA). Variables are presented as Mean ± standard deviation or number (percent). Serum levels of oxidative/lipid profile markers were outcome variables, which had normal distributions. Independent samples t-test was employed for group comparisons as indicated. Linear regression was used for data modeling. Finally a receiver operating characteristics (ROC) curve was generated testing Hcy and LCAT activity for the diabetes status as the state variable. Sensitivity and specificity were determined for multiple Hcy and LCAT activity cut-off values. *P* values less than 0.05 were considered statistically significant.

3. RESULTS

3.1 Baseline Characteristics

82 subjects were enrolled in this cross-sectional study. Demographic and biochemical data of patients and healthy subjects are illustrated in Table 1. No differences were found in the studied variables between males andfemales in patients or control groups. Mean levels of waist, BMI, LDLC, HDL-C, systolic blood pressure and diastolic blood pressure levelswere similar between patients and controls.

As expected, FBS, HbA1c and triglyceride were significantly higher in patients with diabetes in comparison to control group.

Lecithin: cholesterol acyltransferase (LCAT) activity of patients (68.21±15.25μmol/lit/hour) was significantly lower than that of control group. (87.1±6.4μmol/lit/hour) ($p<0.001$) Homocysteine level in patients (13.4±4.12 μmol/lit) was higher than control subjects (12.6±4.10μmol/lit). Moreover, there was a gender difference in homocysteine level, with male subjects having higher homocysteine level (26.4.2±12.77 μmol/L in males vs. 19.3±8.39μmol/L in females).

Table 1. Demographic and biochemical variables in patients with diabetes and healthy controls

Parameters	Patient (n=41)	Control (n=41)	P value
Age(years)	49.9±6.8	49.5±6.9	NS
Duration of diabetes (years)	4.28	0	NS
Male (n, %)	22 (53%)	21(51%)	NS
Female(n,%)	19(46%)	20(48%)	NS
BMI(kg/m^2)	27.2±4.5	27.2±4.1	NS
SBP(mmHg)	120.5±12.9	123.1±14.6	NS
DBP(mmHg)	74.2±6.7	80.1±11.9	NS
FBS(mg/dL)	205.4±73.6	90.3±30.6	<0.001
TG(mg/dL)	199.9±121.9	119.6±40.9	<0.001
CHOL(mg/dL)	195.4±33.4	186.9±30.0	NS
HDL(mg/dL)	42.1±8.5	47.8±10.8	NS
LDL(mg/dL)	129.2±20.6	125.2±24.4	NS
Creatinine (mg/dL)	0.9±0.2	0.8±0.18	NS
HbA1c(%)	8.2±2.2	5.1±0.5	<0.001
HbA1c (µmol/mol)	64.3±26.6	35.1±11.00	<0.001
LCAT activity (µmol/lit/hour)	68.2±15.2	87.1±6.4	<0.001
Homocysteine (µmol/L)	13.4±4.12	12.6±4.10	<0.001

Demographic and biochemical variables in patients with diabetes and healthy subjects. For each variable, mean levels were compared between patients with diabetes and control subjects, and P values of t-tests evaluating the difference of means were measured. Values are mean ± SD. The column of "P value" is presented when comparing patients with type 2 diabetes and controls. BMI : body mass index; DBP: diastolic blood pressure (mmHg); SBP: systolic blood pressure (mmHg); FBS: fasting blood sugar (mg/dl); TG: triglyceride (mg/dl); CHOL: total cholesterol (mg/dl); HDL: high-density lipoproteins (mg/dl); LDL: low-density lipoproteins (mg/dl); Cr: plasma creatinine (mg/dl); HbA1c:hemoglubin A1c (%)(mmol/mol); Hcy: homocysteine (µmol/L); LCAT: Lecithin Cholesterol Acyltransferase ; NS: not significant

3.2 Determinants of Homocysteine and LCAT Activity Levelsin Patients with Diabetes and Normal Subjects

We first examined the correlation of homocysteine (Hcy) and LCAT in both patients with type 2 diabetes and normal subjects. There were no significant correlation between plasma Hcy concentrations and LCAT activity in patients ($r = -0.071$, $p = 0.66$). The same were true for control subjects.

Univariate correlations and multiple regression analyses were performed to establish the principal determinants of the plasma concentrations of Hcy, LCAT activity and HDL in the patients with diabetes and control subjects.
The parameters that reached significant levels of univariate correlation with LCAT activity in patients were serum cholesterol, triglyceride and HDL-C concentrations (r=0.373, r=0.384, and r=0.227, respectively), whereas that for the Hcy was serum urea concentrations(r=0.488) (Table 2).

Linear Regression and correlation analysis revealed that there is no significant correlation between Hcy and HDL in patients and control subjects. We stratified the patients with type 2 diabetes and controls into groups of high and low HDL-C (cut point 40 mg/dl) levels. Stratifying both patients and subjects, revealed that HDL levels above 40 mg/dl is able to predict Hcy levels significantly. However, there was no significant difference between coefficients of determination in control subjects with HDL levels above 40 mg/dl (n=22) compared to patients with HDL levels above 40 mg/dl (n=19) (Fig. 1). After adjustment for various confounder factors, we found HDL to be the best predictor among multiple markers for Hcy levels in control subjects and patients with HDL levels above 40 mg/dl.

3.3 Serum HDL and HbA1c Levels Affects LCAT Activity and Hcy Correlations

To shed light on the reason for observed differences in Hcy correlation with HDL in different HDL concentrations and addressing possible correlations of LCAT activity and Hcy, it was supposed to determine mean levels of LCAT activity and Hcy and their correlation at different HDL levels.

Table 2. Hcy level and LCAT activity in patients with diabetes and control subjects

	Biochemical marker	LCAT activity		Hcy	
		r	*p*	r	*p*
Univariate correlations	**HDL**				
	Control				
	Patients	0.23	0.012		
	Urea				
	Control				
	Patients			0.49	0.02
	CHOL				
	Control				
	Patients	0.37	0.02		
	TG				
	Control				
	Patients	0.38	0.01		

Hcy level and LCAT activity in patients with diabetes and control subjects. Note that HDL, CHOL and TG establish positive correlation with LCAT activity in patients. While urea level was the only factor having positive correlation with Hcy levels. TG: triglyceride (mg/dl); CHOL: total cholesterol (mg/dl); HDL: high-density lipoproteins (mg/dl); Hcy: homocysteine (µmol/L); LCAT: Lecithin Cholesterol Acyltransferase

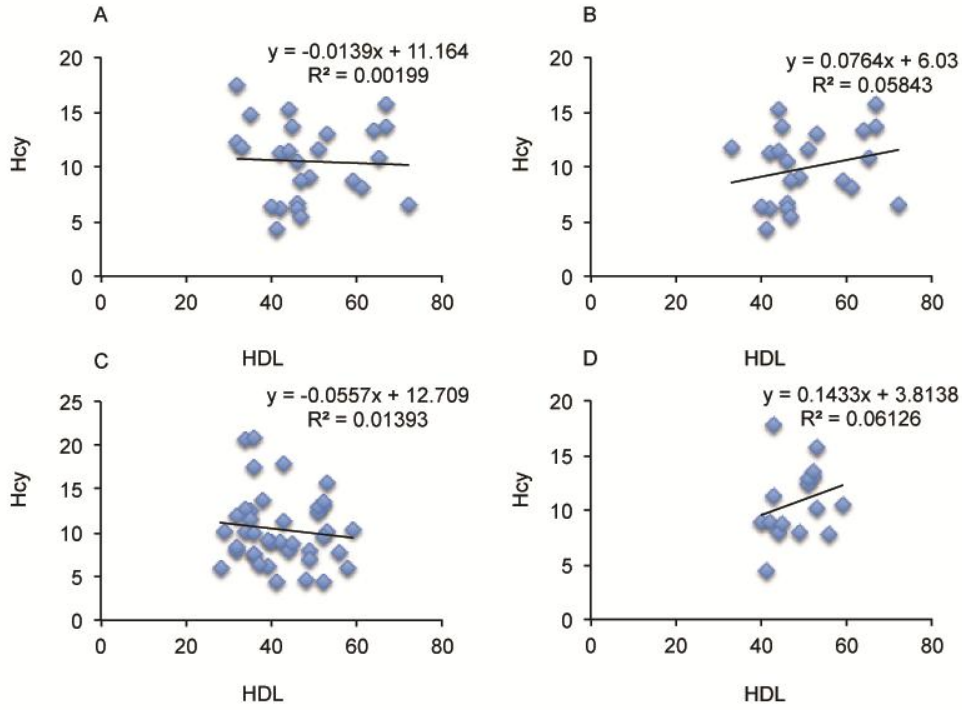

Fig. 1. Scatter plot demonstrating linear regressions of Hcy with HDL in patients with type 2 diabetes mellitus before and after stratification based on serum HDL levels. Each data point represents 1 patient. A. Correlation of HDL-C concentration vs plasma levels of Hcy in control subjects. B. Correlation of HDL-C concentration vs plasma levels of Hcy in control subjects with HDL levels higher than 40 mg/dl. C. Correlation of HDL-C concentration vs plasma levels of Hcy in patients. D. Correlation of HDL-C concentration vs plasma levels of Hcy in patients with HDL leves higher than 40 mg/dl

HDL: High-Density Lipoprotein, Hcy: Homocysteine

Patients were stratified based on their HDL-C levels into 4 groups; HDL-C≥ 35 mg/dl, HDL-C≥40 mg/dl, HDL-C≥45 mg/dl and HDL-C≥ 50 mg/dl.

Patients with HDL-C<40 mg/dl showed no correlation between LCAT and Hcy. HDL-C levels higher than 50, 45 and 40 mg/dl lead to significant positive correlations between LCAT activity and Hcy (Pearson correlation coefficient: 0.784, 0.772and 0.5, respectively). Interestingly, patients with higher HDL-C levels (above 45 and 50 mg/dl) had a stronger positive correlation in comparison to patients with HDL-C≥40 mg/dl (Table 3).

Subsequently, patients were divided based on their HbA1c level into 2 groups; group 1: ≤ 53.0 mmol/mol (n=17), group 2: > 53.0 mmol/mol (n=24).

We observed a strong negative correlation between homocysteine (Hcy) level and LCAT activity within patients with diabetes with HbA1c below 53.0 mmol/mol (Pearson correlation: -0.601*P* value: 0.02). But in patients with HbA1c> 53.0 mmol/mol, there were no significant correlation between LCAT activity and Hcy levels.

3.4 ROC Curve Analysis Revealed that LCAT Activity and Hcy Levels are not able to predict Diabetes

In order to evaluate Hcy and LCAT activity capability in predicting diabetes and diabetes related oxidative stress, we derived a ROC curve for having diabetes with Hcy and LCAT activity as the test variables. Both of these variables showed an area under curve of below 0.5 and therefore being insignificant in predicting diabetes and its associated oxidative stress. In contrast to glycemic control related variables, Hcy and LCAT activity were able to predict lipid profile related variables significantly with an area under the curve above 0.5 (Fig. 2).

3.5 LCAT Activity Predicts Hyperhomocysteinemia in Patients with Type 2 Diabetes Mellitus

We have derived ROC curves for LCAT activity in predicting parameters associated with cardiovascular events. Hcy levels above 10.5 µmol/litis usually considered as a cut-off point associated with these events. Fig. 3 displays the ROC curves for hyperhomocysteinemia as state variable and LCAT activity as test variable. Both are skewed from 45 diagonal, the one for patients with diabetes being more shifted up and to the left (area under the curve: 0.67) than for control subjects (area under the curve: 0.57). The cutoffs with best equilibrium between sensitivity and specificity, approached and it was 86.7 µmol/lit/hour for control subjects (with 60% and 59.3% sensitivity and specificity, respectively) and 81.3 µmol/lit/hour for diabetic patients (with 60% and 73% sensitivity and specificity, respectively).

Table 3. Serum LCAT activity and Hcy levels in patients stratified based on HDL level

Biochemical marker	Correlation coefficient	*p* value
HbA1c <= 53.0 mmol/mol	-0.601	0.023
HDL >= 35 mg/dl(n=31)	0.251	0.167
HDL >= 40 mg/dl(n=19)	0.5	0.021
HDL >= 45 mg/dl(n=12)	0.772	0.001
HDL >= 50 mg/dl (n=8)	0.784	0.004

Serum LCAT activity and Hcy levels in patients. Plasma of 82 fasted patients and control subjects were collected and analyzed for LCAT activity and Hcy levels. Correlation coefficient and p value based on changes in serum biochemical markers were calculated. Note that the highest correlation is established for HDL levels above 45 and 50 mg/dl. However, HbA1C levels below 53.0 mmol/mol, maintains significant negative correlation between LCAT activity and Hcy level

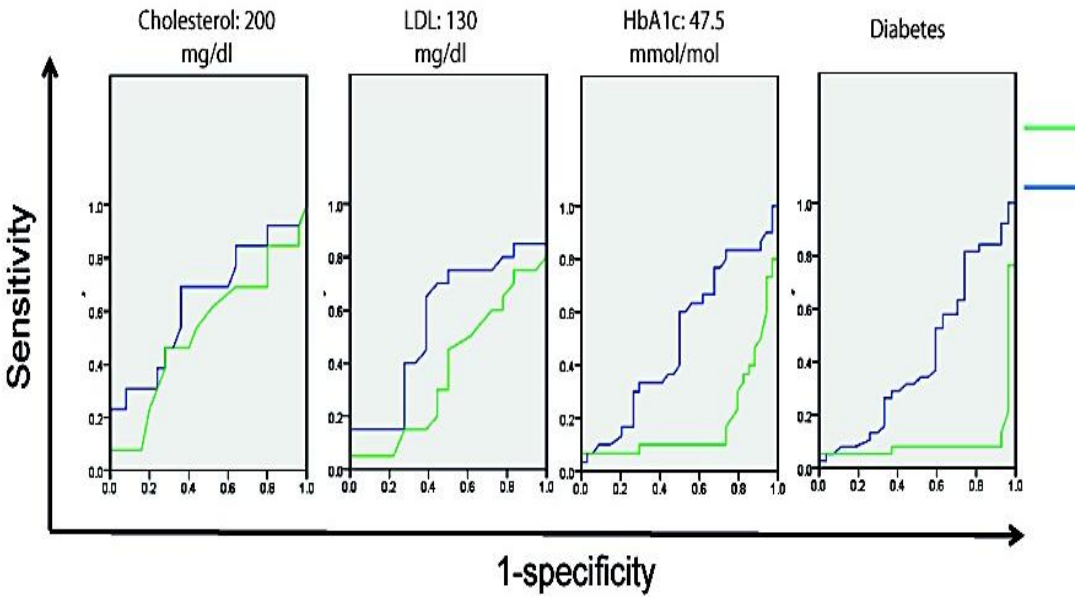

Fig. 2. Receiver operating characteristic curve statistics comparing LCAT activity and homocysteine (Hcy) in predicting principal components of dyslipidemia and glycemic control variables. Cutoffs for variables are as follows: Cholesterol ≥ 200 mg/dl, LDL ≥ 130 mg/dl, HbA1c ≥ 47.5 mmol/mol

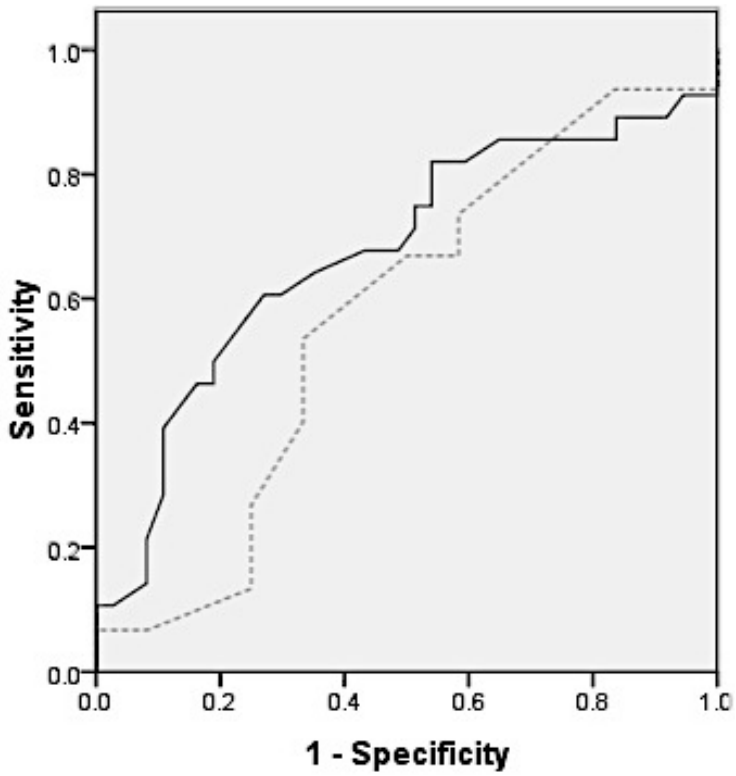

Fig. 3. Receiver operating characteristic curve statistics comparing accuracy of LCAT activity in predicting homocysteine in patients with diabetes (black line) vs. control subjects

4. DISCUSSION

Many epidemiological studies have confirmed that elevated plasma levels of homocysteine (Hcy) are associated with an increased risk of coronary events, stroke, and atheroembolic events as an independent risk factor [21]. Serum Hcy level is increased in patients with type 2diabetes [7], inducing oxidative stress in these patients [5,22].

Results from *in vivo* and *in* vitro studies have led to rapid progress in defining the pathophysiological consequences of hyperhomocysteinemia. However, consequences of increased Hcy level in human studies are conflicting. Some studies reported reduced plasma HDL-C levels in hyperhomocysteinemia, [13,23] while others showed increased concentration [14] or no change with Hcy alterations [24]. One of the possible reasons for these conflicting data originated from the difference in *in vitro* with *in vivo* or human studies. For example, most of Hcy's inhibitory effects on HDL concentration and expression of HDL components such as apoA-I were observed in supra-physiologic concentrations of Hcy [25].

Linear Regression and correlation analysis revealed that there is no significant correlation between Hcy and HDL in patients and control subjects. We stratified the patients with type 2 diabetes and controls intogroups of high and low HDL-C (cut point 40 mg/dl) levels. Stratifying both patients and subjects, revealed HDL to be the best predictor among multiple markers for Hcy levels in control subjects and patients with HDL levels above 40 mg/dl.

Cell-derived free cholesterol is esterified by lecithin: cholesterol acyltransferase (LCAT), allowing pre-HDL-C to become a migrating HDL-C and detectable by liver surface receptors. LCAT is able to facilitate the removal of excess cell cholesterol. Therefore it was considered as an anti-atherosclerotic agent. However, recent studies showed that higher LCAT activity levels not associated with lower cardiovascular disease risk and even it may attenuate cardio-protection associated with higher HDL levels [26]. Moreover, LCAT activity has been found to be associated with attenuation in HDL's anti-oxidative capacitates [19,20] Therefore we investigated whether there is a correlation between homocysteine as a known risk factor for vascular disease and LCAT activity in patients with type 2 diabetes mellitus as an indirect index for HDL's anti-inflammatory capacities. Hyperhomocysteinemia is believed to be associated with decreased anti-atherosclerotic properties of circulating HDL-C particles in animal models [23,27]. To shed light on incongruity regarding increased HDL concentration parallel with increased atherosclerosis in increased Hcy states [13,14,24], it was supposed to determine mean levels of LCAT activity,as an indirect indicator of attenuated anti-oxidative capacity of HDL, and Hcy and their correlation. Grossly analyzing, Hcy level had no correlation with LCAT activity in patients or in control group.Consistent with observations regarding Hcy and HDL correlations in HDL levels above 40 mg/dl, these HDL levels established positive correlation between Hcy and LCAT activity (Table 2). These observations might suggest that Hcy could be associated with HDL's decreased anti-inflammatory capacitates more than its concentrations. Receiver operating characteristics (ROC) curves was generated testing LCAT activity for the hyperhomocysteinemia as the state variable for both control subjects and patients with diabetes.Although the one for patients with diabetes being more shifted up and to the left, but the cutoffs with best equilibrium between sensitivity and specificity were almost similar between these two groups. It reflects the predictive ability of LCAT activity for hyperhomocysteinein levels above 81.3-86.7µmol/lit/hour.

Next, we wondered whether positive correlation between LCAT activity and Hcy level in patients with diabetes is a finding related to chronic oxidative state or not. Therefore, we stratified our study group into high and low HbA1c (cut-off value 53.0 mmol/mol), the latter one considered as low oxidative state. Interestingly, we observed appearance of a negative correlation between LCAT activity and Hcy levels in this in this low HbA1c, and therefore low oxidative, milieu. But the same trend was not observed in patients with HbA1c> 53.0 mmol/mol.

A ROC curve was generated testing Hcy and LCAT activity as test variables for the diabetes status as the state variable. None of these two serum variables were able to predict glycemic control status as their area under curve level was below 0.5.

One of the problems in our study was a low number of patients with diabetes with HDL-C levels above 40 mg/dl. In addition, in the study

assessment of HDL was limited to HDL's cholesterol content and may not be the best measure of antioxidant function.However, HDL-C, the commonly available measure could have clinical relevance in assessment of possible oxidative capacity in patients with diabetes. In addition, direct measurement of HDL's anti-inflammatory capacities *in vitro* might help us to further validate our data.

Further *In vitro* and *In vivo with* studies are required to confirm our observations. Moreover, a larger study population will help in further validation of findings of this study.However, confirmation of a central role for HDL in the pathophysiology of hyperhomocysteinemia also might help to provide an explanation for the failure of several recent clinical trials to demonstrate a benefit of homocysteine-lowering therapy in prevention of cardiovascular complications of increased Hcy levels. In addition, it might help in directing future therapeutic studies in hyperhomocysteinemia towards HDL increasing approaches.

5. CONCLUSION

LCAT activity could be considered as one of the factors predicting hyperhomocysteinemia and therefore indirectly associated with cardiovascular events and it is more specific and sensitive in patients with diabetes compared to control subjects. However neither Hcy and nor LCAT activity, are able to predict diabetes and/or diabetes associated oxidative stress. These factors might be more associated with diabetes related dyslipidemia.

COMPETING INTEREST

Authors have declared that no competing interests exist.

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