Lipoprotein associated phospholipase A2 activity & its correlation with oxidized LDL & glycaemic status in early stages of type-2 diabetes mellitus

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Background & objectives: Lipoprotein associated phospholipase A_2 (Lp-PLA_2) is an important risk predictor of coronary artery disease (CAD). This study was aimed to evaluate Lp-PLA_2 activity and oxidized low density lipoprotein (oxLDL) in newly diagnosed patients of type 2 diabetes mellitus and to determine the correlation of Lp-PLA_2 activity with oxLDL and plasma glucose levels.

Methods: Blood samples were collected in patients with newly diagnosed type 2 diabetes (n=40) before any treatment was started and healthy controls (n=40). These were processed for estimating plasma glucose: fasting and post prandial, ox LDL, and Lp-PLA2 activity. The parameters in the two groups were compared. Correlation between different parameters was calculated by Pearson correlation analysis in both groups.

Results: Lp-PLA_2 activity (24.48 ± 4.91 *vs* 18.63 ± 5.29 nmol/min/ml, *P<0.001*) and oxLDL levels (52.46 ± 40.19 *vs* 33.26 ± 12.54 µmol/l, *P<0.01*) were significantly higher in patients as compared to those in controls. Lp-PLA_2 activity correlated positively with oxLDL in both controls (r=0.414, *P<0.01*), as well in patients (r=0.542, *P<0.01*). A positive correlation between Lp-PLA_2 activity and fasting plasma glucose levels was observed only in patients (r=0.348, *P<0.05*).

Interpretation & conclusions: Result of this study implies that higher risk of CAD in patients with diabetes may be due to increase in Lp-PLA_2 activity during the early course of the disease. A positive correlation between enzyme activity and fasting plasma glucose indicates an association between hyperglycaemia and increased activity of Lp-PLA2. This may explain a higher occurrence of CAD in patients with diabetes. A positive correlation between oxLDL and Lp-PLA2 activity suggests that Lp-PLA2 activity may be affected by oxLDL also.

Key words Lipoprotein associated phospholipase A_2 - oxidized LDL - plasma glucose - platelet activating factor-acetyl hydrolase - type 2 diabetes mellitus

Diabetes is a chronic metabolic condition leading to microvascular and macrovascular complications resulting in considerable morbidity and mortality. India is currently experiencing an epidemic of type 2 diabetes mellitus (T2DM) and has a large number of diabetic patients causing considerable economic burden on the country[1]. Patients with diabetes are at a two to four-fold increased risk of cardiovascular disease (CVD)[2]. However, the mechanism that predisposes these patients to increased risk of CVD is poorly understood. Inflammatory processes have been increasingly recognized to play a role in pathogenesis of both diabetes and heart disease, and may offer a biological link between the two diseases[3]. Various circulating markers of inflammation have been extensively evaluated for their role as risk predictors of cardiovascular disease[4]. Amongst these markers lipoprotein associated phospholipase A2 (Lp-PLA$_2$) has attracted considerable interest in the last decade[4,5]. Many prospective studies have also indicated that Lp-PLA2 is an independent predictor of coronary artery disease (CAD)[6,7].

Lp-PLA$_2$ is a subtype of the phospholipase A$_2$ superfamily, a family of enzymes that hydrolyze phospholipids. Lp-PLA$_2$, also known as platelet activating factor acetylhydrolase (PAF-AH), is a 50-kDa Ca^{2+}-independent phospholipase[7]. This enzyme is transported in plasma predominantly (80%) in association with low density lipoprotein (LDL) and a smaller fraction (20%) is transported with high density lipoprotein (HDL)[7,8]. But this distribution is believed to be altered in type 2 diabetes[9]. Though some studies have tried to provide evidence that this enzyme might be antiatherogenic owing to its anti-inflammatory effects, most other studies have attributed inflammatory role to Lp-PLA$_2$[8,10,11]. Moreover, several other reports suggest that Lp-PLA$_2$ plays a critical role in the development of atherosclerosis and its clinical sequelae[12,13]. Lp-PLA$_2$ is upregulated in atherosclerotic plaques and is strongly expressed in macrophages within the fibrous cap of rupture prone lesions[12,13]. Atherogenicity of Lp-PLA$_2$ is due to its action on oxidized LDL (oxLDL)[14]. The hydrolysis of oxLDL by Lp-PLA$_2$ produces the proinflammatory and atherogenic by-products[14].

In patients of type 2 diabetes, there is a preponderance of atherogenic dense low-density lipoprotein (dLDL) as well as oxLDL[15]. The dLDL is more liable to be oxidised and can be easily taken up by macrophages in extravascular spaces resulting in atherogenesis[16]. Although some researchers have estimated the activity of this enzyme in type 1 and type 2 diabetes[17-20], we did not come across any study specifically assessing the activity in early stages of the disease or the effect of glycemic status of the patients on this enzyme. Therefore, this study intended to evaluate the activity of Lp-PLA$_2$ in newly diagnosed patients of type 2 diabetes mellitus, and the correlation of this enzyme with oxLDL and plasma glucose levels was also investigated.

Material & Methods

The study was conducted in the departments of Biochemistry and Medicine, University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India, for 17 months (January 2011-June, 2012), after obtaining ethical clearance from the institutional ethical committee for human studies. This was a case-control study in which patients of T2DM, who were diagnosed for the first time, were recruited consecutively from the diabetic clinic of the department of Medicine. Controls comprised relatives, spouses, or friends of the patients and staff members of the institute and were matched for age and sex. All the participants were more than 35 years of age and were diagnosed on the basis of American Diabetes Association (ADA) criteria[21]. An informed written consent was obtained from the participants before recruiting them in the study. A careful history to establish the time of onset of the symptoms like polyuria, nocturia, polydipsia, weakness, loss or gain of weight/appetite was taken. The patients who had any of these symptoms for the duration of less than six months were included in the study. Exclusion criteria for this study were: presence of thyroid disorders, renal dysfunction, liver dysfunction, previous history of diabetes mellitus and previous or present history of cardiovascular disease as assessed from history of chest pain, stroke and ECG. The patients on any kind of medications were also excluded from the study.

Anthropometry: Weight was measured using a digital scale with sensitivity of 0.1kg, height was measured to the nearest 0.1cm using wall mounted scale. Body mass index (BMI) was calculated as weight in kg divided by squared height (m^2). Waist-hip ratio (WHR) was calculated as ratio of waist circumference measured at the level of umbilicus after expiration to hip circumference (measured as maximal horizontal circumference at the level of the buttocks)

Blood collection and biochemical analysis: Blood samples (5 ml) were collected before initiating the

treatment in patients. Venous blood was collected from all participants after an overnight fast and after 2 h of 75 g glucose load by standard protocol to assess for glycaemic status[21]. Fasting blood samples were used to analyse HbA1c and biochemical parameters, *i.e.* for lipid profile parameters (total cholesterol, HDL-C, LDL-C, VLDL-C and triglycerides), insulin levels, renal functions (serum urea, creatinine, electrolytes) and liver functions [serum bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP)]. Serum samples collected for estimation of oxLDL were frozen at -80°C and were analysed within one month. For plasma samples blood was collected in EDTA vials and separation of plasma was done by centrifugation at 4°C for 10 min. The plasma samples were stored at -80°C and analysed later for activity of Lp-PLA$_2$.

Plasma glucose, serum cholesterol and serum triglycerides were estimated using standard colorimetric enzymatic methods on Olympus AU400, Japan. Plasma glucose was measured using glucose oxidase enzyme[22]. Serum cholesterol estimation was carried out by using cholesterol esterase and cholesterol oxidase enzymes[23]. Serum triglycerides were determined after enzymatic hydrolysis with lipase and production of H$_2$O$_2$ by glycerol kinase and glycerol phosphate oxidase[24]. H$_2$O$_2$ produced in all three methods converts 4-aminoantipyrine to red coloured quinoneimine dye in presence of peroxidase enzyme. HDL-C levels were analysed by direct method according to standard protocol[25]. Cholesterol esterase and cholesterol oxidase selectively oxidise cholesterol of HDL fraction to produce coloured dye with H$_2$O$_2$. LDL-C was calculated by Freidwald formula[26]. HbA1c was estimated using HPLC method (Bio-Rad D-10 Hemoglobin Testing System) and ox-LDL was estimated by the method of Ahutopa *et al*[27]. The intra and inter- assay coefficients of variation (CV) for this method were 4.4 and 4.5 per cent, respectively. Serum insulin was estimated by ELISA method (DiaMetra, Italy) according to the manufacturer's protocol. The intra- and inter- assay CV for this method were 5 and 10 per cent, respectively. Insulin resistance was assessed using homeostasis model assessment-estimated insulin resistance (HOMA-IR model), *i.e.* serum fasting insulin (μU/ml) X fasting glucose (mmol/l)/22.5[28]. Lp-PLA$_2$ activity was measured by using spectrophotometric assay (Cayman Chemicals MI, USA) which used thiol derivative of platelet activating factor (PAF) as a substrate. Upon hydrolysis of acetyl thioester bond at the *sn*-2 by Lp-PLA$_2$, free thiols are detected using 5,5'- dithio-*bis*-(2-

nitrobenzoic acid). Intra-assay CV of this method was 3.5 per cent and inter-assay CV was 10 per cent. The power of the study was 0.904 when calculated with true difference in patient and control means of 0.0034 and a standard deviation of 0.0034 and a standard deviation of 0.0046 taken from a previous study[20]. The Type 1 error probability associated with this test of this null hypothesis was 0.05.

Statistical analysis: Statistical analysis was done using Students' t test for comparison between the two groups using SPSS version 12 (Chicago, II, USA). Relationship between variables was assessed by Pearson correlation analysis in both groups. Multiple linear regression analysis was used to assess the effect of BMI, WHR and dyslipidaemia keeping Lp-PLA$_2$ activity as dependent variable.

Results

A total of 80 individuals participated in the study. The clinical and biochemical characteristics of the study population are shown in the Table. Considering the cut-off limits of WHR for abdominal obesity as ≥0.85 for females and ≥0.95 for males, 75 per cent of the patients and 48 per cent of the controls were found to be obese[29]. However, WHR in the two groups was not significantly different. BMI in the patients was significantly higher (*P*<0.05) than in controls. Further, 80 per cent of controls and 92.5 per cent of the patients had dyslipidaemia. Presence of dyslipidaemia was diagnosed when a subject had one or more of the following criteria *(i)* LDL cholesterol of ≥2.58 mmol/l; *(ii)* non-HDL cholesterol of ≥3.36 mmol/l; *(iii)* plasma triglycerides of ≥1.69 mmol/l; and *(iv)* HDL cholesterol of ≤1.03 mmol/l[30]. There was no significant difference between the lipid profile of patients and controls except in HDL-cholesterol. Patients of type 2 diabetes had significantly low (*P*<0.05) level of HDL-cholesterol as compared to controls. According to the cut-off values given by the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation and Treatment of High Blood Pressure, 62.5 per cent patients and 10 per cent controls were categorised as hypertensive[31]. None of the participants was smoker and only one patient consumed alcohol occasionally. ox-LDL levels were significantly higher (*P*<0.01) in patients as compared to controls. Activity of Lp-PLA$_2$ was also found to be significantly (*P*<0.001) greater in patients in comparison to that in controls. Patients of type 2 diabetes mellitus had significantly higher (*P*<0.05) serum insulin levels as compared to controls (Table).

Table. Clinical, anthropometric, and biochemical parameters of patients and controls		
	Controls (n=40)	Patients (n=40)
Age (yr)	44.4 ± 7.96	46.03 ± 6.70
Male: Female ratio	20:20	18:22
Body mass index (kg/m^2)	24.98 ± 3.33	27.70 ± 4.50[*]
Waist hip ratio	0.90 ± 0.08	0.92 ± 0.08
Plasma glucose- fasting (mmol/l)	4.48 ± 0.73	9.78 ± 2.94[***]
Plasma glucose- post-prandial (mmol/l)	5.85 ± 1.15	16.25 ± 4.54[***]
HbA1c (%)	5.34 ± 0.62	10.81 ± 2.59[***]
Total cholesterol (mmol/l)	5.10 ± 0.94	4.91 ± 0.79
HDL-cholesterol (mmol/l)	1.09 ± 0.20	0.98 ± 0.21[*]
LDL-cholesterol (mmol/l)	3.10 ± 0.90	3.08 ± 0.86
Triglycerides (mmol/l) (geometric mean by log transformation)	3.72	4.19
Ox-LDL (μmol/l)	33.26 ± 12.54	52.46 ± 40.19[**]
Lp-PLA$_2$ activity (nmol/min/ml)	18.63 ± 5.29	24.48 ± 4.91[***]
Serum Insulin (μIU/ml)	9.02 ± 2.82	12.08 ± 5.76[*]
HOMA-IR	1.94 ± 0.54	5.42 ± 2.21[***]

Values are expressed in mean ± SD
LDL, low density lipoprotein; HDL, high density lipoprotein; Ox-LDL, oxidised low density lipoprotein; Lp-PLA2, lipoprotein associated phospholipase A2; HOMA-IR, homeostesis model assessment-insulin resistance
P*<0.05, **<0.01, ***<0.001 compared with controls

Total Lp-PLA$_2$ activity correlated positively with plasma glucose levels (r=0.348, *P*<0.05) (Fig. 1) and negatively with serum insulin levels (r=-0.500, P=0.001) (Fig. 2) only in patients. When correlation analysis was carried out between insulin resistance and Lp-PLA$_2$ activity and oxLDL for all 80 participants, it was observed that there was significant correlation of insulin resistance (HOMA-IR) with Lp-PLA$_2$ activity (r=0.252, *P*<0.05) and oxLDL levels (r=0.451, *P*<0.001). No significant correlation was observed between enzyme activity and HOMA-IR when analysed for both groups separately (r= -0.130 in patients and r= 0.023 in controls). oxLDL was also not found to correlate significantly with HOMA-IR in any of the groups (r= 0.097 in patient group and r= 0.054 in control group). No significant correlation of the enzyme activity was observed with HbA1c in both the groups (r= 0.162, in patients and r= 0.022, in controls). Lp-PLA$_2$ activity had a positive correlation with oxLDL in both patients (r= 0.542, *P*<0.001) and controls (r= 0.414, *P*<0.01) (Fig. 3). oxLDL correlated positively with fasting plasma glucose (r=0.330, *P*<0.01) levels as well as with post-prandial plasma glucose levels (r=0.395, *P*<0.001) in all subjects (n=80). No significant correlation was observed between WHR and enzyme activity in any of the two groups (r= 0.09 in patients and r=0.03 in controls). Correlation studies did not reveal any significant association between BMI and the enzyme activity in any of the two groups (r= -0.161 in patients and r= 0.057 in controls).

Multiple regression analysis was carried out to adjust for BMI, WHR and presence of dyslipidaemia keeping Lp-PLA$_2$ as a dependent variable. The analysis revealed that Lp-PLA$_2$ activity was positively associated with newly diagnosed diabetes [0.006 (95%CI: 0.004 to 0.008), *P*<0.001 standardized coefficient=0.506, adjusted R-square of model= 0.229].

Discussion

In the present study Lp-PLA2 activity was found to be raised significantly in patients of type 2 diabetes during the early stages of disease. Since this enzyme has been recognised as an important risk predictor for CAD[4-6], findings of our study may indicate a higher risk of CAD in them. oxLDL correlated positively with activity of Lp-PLA$_2$ in all subjects. We thus propose that oxLDL being substrate of Lp-PLA$_2$ may affect the activity of this enzyme. In diabetes, modified LDL levels are elevated which can readily undergo oxidation[15,16]. Lp-PLA$_2$ hydrolyses oxidised phospholipids of oxLDL
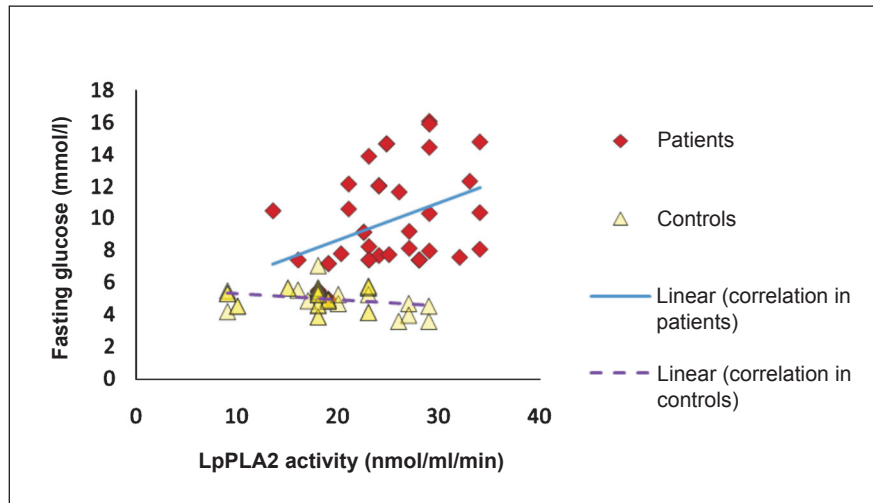
Fig. 1. Correlation between Lp-PLA$_2$ activity and fasting plasma glucose levels in patients and controls .
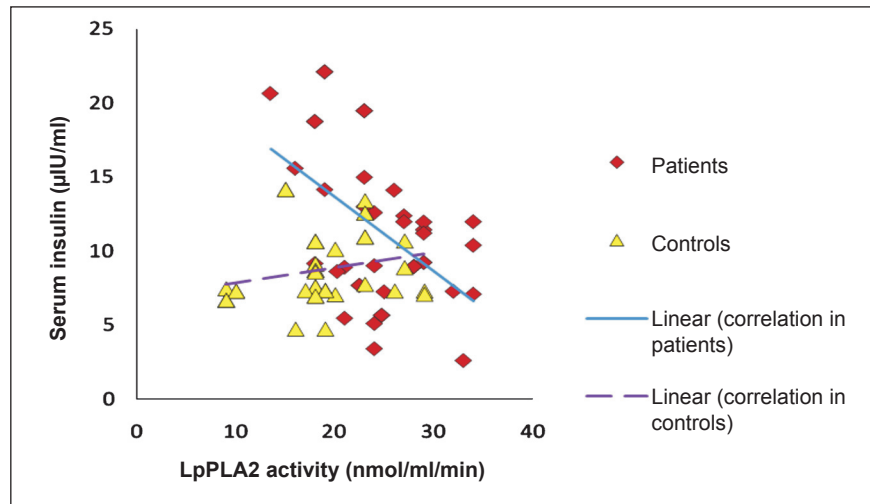

Fig. 2. Correlation between Lp-PLA$_2$ activity and fasting serum insulin levels in patients and controls .
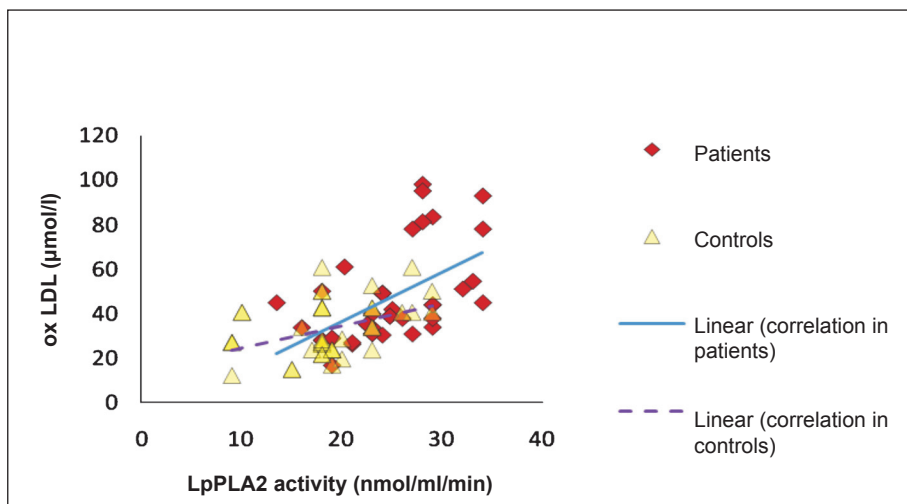

Fig. 3. Correlation between Lp-PLA$_2$ activity and ox LDL levels in patients and controls.

to produce lysophosphatidylcholine (LysoPC) and oxidized non esterified fatty acids (oxNEFA)[14]. These two products are proinflammatory and atherogenic and are important contributors to the risk of CAD. In addition, a positive correlation was observed between enzyme activity and fasting glucose in diabetes group. Therefore, it appears that hyperglycaemia may also affect the activity of this enzyme. However, a positive correlation between oxLDL and plasma glucose levels indicates that the effect of hyperglycaemia may also be mediated through increase in oxLDL levels. Thus, there is a probability that early treatment of hyperglycaemia may result in reduction of enzyme activity and decrease the risk of CAD, although prospective studies are needed to ascertain this.

A few other studies conducted on patients of long standing diabetes assessed Lp-PLA$_2$ activity and tried to establish its association with CVD[17-19]. Two such studies revealed that the activity of the Lp-PLA$_2$ increased in these patients[18,19]. In one of these it was observed that type 2 diabetic patients in whom Lp-PLA$_2$ activity was elevated were also more likely to develop CAD than those without elevated levels[18]. On the contrary, a more recent prospective case control study revealed that there was no significant difference in the activity of Lp-PLA$_2$ and its association with CAD between diabetic and non diabetic groups[17]. Thus, the two studies have given contrasting results[17,18]. None of these studies explored the correlation of enzyme activity with plasma glucose and oxLDL concentration. Though Hatoum *et al*[18] observed that HbA1c correlated positively with Lp-PLA$_2$ activity only in females, no observation was made regarding correlation with plasma glucose. It has been demonstrated that statins (lovastatin, fluvastatin) reduce the activity of Lp-PLA$_2$ enzyme in patients with diabetes[32,33]. Thus, hypoglycaemic and hypolipidaemic drugs may be acting as confounding factors in these studies[17,18]. Since we observed Lp-PLA$_2$ activity correlated positively with plasma glucose levels and oxLDL levels, it appears that these two parameters may be acting as additional confounding factors in earlier two studies[17,18]. A significantly higher Lp-PLA$_2$ activity was observed in the newly diagnosed patients in our study as shown earlier[18]. Thus, it seems probable that after an initial rise in the enzyme activity a steady state is achieved later in the disease. This could be a consequence of a change in the metabolic milieu due to medical intervention or chemotherapeutic agents may directly be modulating the activity of enzyme. An important difference in our study is the inclusion of

patients with a short history of symptoms and who are not on any drugs. This makes our study more relevant in understanding the reason for increased risk of CAD in these patients.

In our study a negative correlation was observed between Lp-PLA$_2$ activity and insulin levels in diabetes patients. This is contrary to the results observed previously by Kudolo *et al*[33] in which enzyme activity correlated positively with fasting plasma insulin levels. However, they used a very small sample size (6 patients). Our study indicated a negative correlation between fasting plasma insulin levels and activity of Lp-PLA$_2$. However, we did not find any correlation between enzyme activity and insulin resistance (calculated by HOMA–IR model) in patients. A larger group size is required to establish the association of insulin resistance and Lp-PLA$_2$ activity.

Apart from diabetes, other inflammatory conditions like obesity also contribute to risk of CAD[29]. BMI is reported to significantly affect Lp-PLA$_2$ activity, although differently in both sexes[29]. In our study even though number of obese individuals was higher in patient group, no significant correlation was observed between WHR or BMI and Lp-PLA$_2$ activity. Additionally, significantly higher Lp-PLA2 activity in newly diagnosed T2 DM patients despite high numbers of obese and dyslipidaemic subjects in controls points to an additional contribution of diabetes over and above obesity and dyslipidaemia. Regression analysis also revealed a strong positive association between diabetes and Lp-PLA2 activity independent of BMI, WHR, and presence of dyslipidaemia. Thus, in our study on Indian population, obesity was not found to be modulating the enzyme activity, at least not in the initial stages of the disease.

As Lp-PLA$_2$ is used as a risk predictor for CAD, it is important that its activity is measured in all patients of diabetes. Since a high activity of this enzyme was observed in the previous studies inspite of the treatment with hypoglycaemics[18,19], it may appear that treatment of diabetes has very little, if any, effect on the activity of this enzyme. However, our conclusion that hyperglycaemia affects the enzyme activity was corroborated by a recently published study which revealed that if the glycaemic status (as assessed by HbA1c) improved, the activity of Lp-PLA$_2$ decreased[34]. Thus, it is ascertained that enzyme activity rises early in the course of diabetes, but maintaining a good glycaemic control may reduce the enzyme activity and

thus decrease the risk of CAD. With the advent of some novel pharmacological inhibitors of this enzyme such as darapladib and varespladib which have emerged as promising therapeutic options for treating patients with coronary artery disease, specific therapeutic intervention for Lp-PLA$_2$ in early stages of diabetes may help to reduce the risk of CAD.[35] However, prospective studies on a large sample size are required to test this hypothesis as well as to establish the effect of hyperglycaemia and different drugs on this enzyme.

There were several limitations of this study. The population size was small and only included patients from eastern region of the National Capital Teritory of Delhi as well as the adjoining area of the neighbouring State. Thus, our study population was not representative of the entire Indian population. We did not analyse our patients gender-wise because of small sample size. The strength of the study was that the treatment naive patients with short duration of diabetes were included.

In conclusion, our study showed higher Lp-PLA$_2$ activity in early stages in type 2 diabetes and an independent association with disease. Since Lp-PLA$_2$ predicts the risk of CAD, it is important to assess Lp-PLA$_2$ activity in all patients of diabetes during early phase of disease. Additionally, hyperglycaemia was found to be associated with higher Lp-PLA$_2$ activity. So it is important that hyperglycaemia is treated early and glycaemic status be maintained. This may reduce the activity of Lp-PLA$_2$ and, therefore, lower the incidence of CAD in diabetes patients. However, further studies are required to establish the effect of hyperglycaemia and duration of disease on this enzyme in a larger sample size.

Acknowledgment

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