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Selective decrease in alpha$_1$-antitrypsin levels in diabetic retinopathy: Could the levels of it be playing a role in the pathophysiology of diabetic retinopathy?

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***Background & objectives*: Type 2 diabetes mellitus (T2DM) is known to induce inflammation and activation of neutrophils causing the release of neutrophil elastase (NE), a pro-inflammatory proteinase. The activity of NE is regulated by endogenous inhibitors alpha$_1$-antitrypsin (α_1-AT) and alpha$_2$-macroglobulin (α_2-MG). Disrupted proteolytic homeostasis in T2DM patients is one of the causes for vascular complications. This study was carried out for evaluating the levels of plasma NE, α_1-AT, α_2-MG and NE-α_1-AT complex to understand their roles in the pathophysiology of diabetic nephropathy (DN) and diabetic retinopathy (DR).**

***Methods*: A total of 240 participants (Control, n=60; T2DM, n=60; DN, n=60; and DR, n=60) were recruited after recording history, clinical examination and laboratory investigations. Retinopathy was confirmed by fundoscopy and nephropathy by urinary albumin excretion and serum creatinine levels. NE was measured using STANA. α_1-AT, α_2-MG and NE-α_1-AT complex were estimated by ELISA.**

***Results*: Baseline clinical and laboratory findings were confirmatory to the study groups. The mean elastase activity was higher (*P*<0.0005) in diabetes groups (T2DM=0.73±0.31, DN=0.87±0.35, DR=0.76±0.41) than controls (0.35±0.20). The levels of α_1-AT were lower in DR (8.77±2.85) than DN (26.26±6.16) and T2DM (41.13±14.06) when juxtaposed with controls (122.95±25.71). The approximate fold decrease of α_1-AT levels was 15 for DR and four for DN compared to controls. The levels of α_2-MG were lowered in T2DM (167.29±30.45), DN (144.66±13.72), and DR (104.67±11.47) than controls (208.87±31.16). The NE-α_1-AT complex levels were: controls (215.83±13.61), T2DM (98.85±23.85), DN (129.26±20.40) and DR (153.25±17.11).**

***Interpretation & conclusions*: Homeostasis of NE, α_1-AT and α_2-MG is disrupted in T2DM, DN and DR. Strikingly reduced levels of α_1-AT observed in DR are indicative of its possible role in the pathophysiology of retinopathy and emphasizes α_1-AT as a plausible therapeutic target.**

Key words Alpha$_1$-antitrypsin complex - α_1-antitrypsin - α_2-macroglobulin - diabetic nephropathy - diabetic retinopathy - inflammation - neutrophil elastase - type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder with an increasing global prevalence and incidence. Hyperglycaemia in diabetes has been implicated in the dysfunction of micro- and macro-

vascular systems leading to long-term damage and failure of various organs[1,2]. The pathogenesis of these vascular complications is complex and recent clinical as well as laboratory findings point to inflammation as one of the critical contributors to these conditions. The hyperglycaemic environment in diabetes induces the stimulation of various pro-inflammatory mediators such as cytokines, chemokines and growth factors which are shown to play a major role in the recruitment and activation of neutrophils at the site of inflammation[3-5].

Activated neutrophils release several serine proteases including neutrophil elastase (NE). These serine proteases are involved in the subsequent inflammatory responses and activate protease-activated receptors on the membrane of immune cells which control the neutrophil chemotaxis and mobility[6-8]. Uncontrolled activity of NE can result in tissue damage by breaking down proteins of the cellular membrane and extracellular matrix. Under physiological conditions, the NE activity is regulated by endogenous inhibitors, alpha$_2$-macroglobulin (α_2-MG) alpha$_1$-antitrypsin (α_1-AT) and secretory leucoproteinase inhibitor. These molecules inactivate the free enzyme by forming irreversible complexes[9-11].

α_1-antitrypsin is the major serine protease inhibitor (SERPINS) in circulation. It is a glycoprotein, synthesised mainly in hepatocytes and secreted into the plasma. As an acute-phase reactant, the levels of α_1-AT have been shown to rise during inflammation, infection and malignant conditions[12]. Further, recent studies suggest that α_1-AT may also possess anti-inflammatory, antiapoptotic, immunomodulatory and antimicrobial effects[13-16]. The anti-inflammatory action of α_1-AT is facilitated by its ability to inhibit key pro-inflammatory molecules as well as serine proteases. Human α_2-MG is a glycoprotein inhibitor synthesised mainly in the liver. It traps and inhibits proteolytic enzymes which are involved in inflammation, homeostasis and immunoregulatory processes[17].

The co-existence of low-grade chronic inflammation in T2DM is well established[18,19]. There are reports indicating the activation of neutrophils in T2DM leading to release of elastase and destruction of tissue architecture[20,21]. Further, decreased levels of α_1-AT have been reported in T2DM patients compared to non-diabetic subjects[22,23]. Studies on the levels of α_2-MG in patients with T2DM have indicated an association of this molecule with the complications of diabetes[24].

Endothelial injury is a major pathophysiological factor underlying microvascular complications. This could be due to excessive proteolytic activity as a result of the homeostatic imbalance between NE, α_1-AT and α_2-MG[25]. However, whether such disproportionate levels of these molecules play a role in the development of complications of diabetes is so far not known. This study was therefore carried out for evaluating the levels of plasma NE, α_1-AT, α_2-MG and NE–α_1-AT complex to understand their roles in the pathophysiology of diabetic nephropathy (DN) and diabetic retinopathy (DR).

Material & Methods

Study design: This was a comparative study carried out between June 2015 and January 2017, in the department of Biochemistry, Sri Devaraj Urs Medical College (SDUMC), a constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka after procuring approval from the Institutional Ethics Committee. A total of 240 participants attending or admitted to the department of Medicine, R. L. Jalappa Hospital and Research Centre, the teaching hospital of SDUMC were included in the study. Informed written consent was obtained from every participant before enrolment in the study. The study protocol was approved by the Institutional Ethical Committee of SDUMC and complied with the Helsinki declaration.

Study participants: The participants in the study were divided into four groups of 60 each, (*i*) healthy non-diabetic individuals, based on fasting blood glucose (FBG) 90-105 mg/dl and glycated Hb (HbA_{1c}) <6.0 per cent comprised the control group; (*ii*) T2DM group comprised of clinically proven and confirmed cases of DM without complications whose FBG was ≥126 mg/dl and HbA_{1c} >6.5 per cent; (*iii*) the DN group encompassed DN diagnosed individuals based on persistent proteinuria (urinary albumin excretion rate >300 mg/24 h; confirmed by dip-stick method) and renal function as reflected by an abnormal serum creatinine level; and (*iv*) the DR group comprised of individuals in whom DR was confirmed by fundoscopic examination and was further grouped as non-proliferative DR (NPDR=40) and proliferative DR (PDR=20) based on severity. The Early Treatment of DR Study Classification (ETDRS) was followed for DR grading[26].

Sample size: The sample size was calculated assuming to detect a difference of 60 per cent in α_1-AT levels

between control and T2DM groups with 80 per cent power and five per cent alpha error. The estimated sample size was 54 in each group. Considering a non-response of 10 per cent, the sample size was rounded off to 60 in each group.

The age of the study groups ranged from 30 to 70 yr. Patients with acute and chronic infections, non-diabetic cases with retinopathy or nephropathy, diabetes patients with cardiac diseases, liver diseases, acute renal failure, malignancy, stroke, chronic obstructive lung disease, history of smoking and participants diagnosed for gestational diabetes mellitus were excluded.

Sample collection: Fasting venous blood (6 ml) was collected under aseptic conditions into tubes containing sodium fluoride (for blood glucose estimation), ethylenediaminetetraacetic acid (for HbA1c, erythrocyte sedimentation rate, total leucocyte count) and sodium heparin (for elastase, α_1-AT and α_2-MG and NE-α_1-AT complex estimation). For C-reactive protein (CRP), renal function and liver function tests, blood samples were collected in tubes without anticoagulants. Samples wherever necessary were centrifuged for 15 min at 3000 rpm within two hours of collection for plasma/serum separation. Routine parameters were analyzed immediately and aliquots were stored at −80°C for estimation of NE and its endogenous inhibitors.

Methods: Estimation of blood glucose, blood urea, serum creatinine and liver function tests were carried out by standard methods using Vitros 250 Dry chemistry analyser (Ortho-Clinical Diagnostics, New York, USA). HbA1c was analyzed by HPLC method using Bio-Rad D-10™ Hemoglobin Testing System (Bio-Rad Laboratories Inc., California, USA). Complete blood count was performed using automatic blood cell counter (Beckman- Coulter, USA). CRP was estimated by rapid latex slide tests (SPAN Diagnostics Ltd. India). Plasma α_1-AT, α_2-MG levels (Immunology Consultants Laboratory, Inc., USA) and NE-α_1-AT complex (Calbiochem, USA) were quantified by ELISA.

Plasma NE was estimated using Succinyl tri-L-alanyl-p-nitroanilide (STANA, Sigma, USA) as substrate as per the procedure described by Bieth *et al*[27]. The assay system comprised of 2 mM of STANA in 0.2M of Tris-HCl buffer *p*H 7.6. The reaction was initiated by the addition of plasma. After 15 min incubation at 37°C, the reaction was stopped by the addition of 1.0 ml of 30 per cent acetic acid. The optical density of p-nitroaniline liberated was measured at 410 nm using UV/VIS Spectrometer Lambda 35 (PerkinElmer Life and Analytical Sciences, Singapore). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µm of p-nitroaniline per unit time under the assay conditions.

Statistical analysis: The data were analysed using SPSS software version 22 (IBM Corp, Chicago, IL, USA). All variables were checked for normal distribution by Shapiro-Wilk test. The results are expressed as mean±standard deviation. For statistical differences in means between the groups, analysis of variance with post-hoc Bonferroni test was used. Pearson's correlation coefficient was used to analyze the correlation between variables in the DR group and the level of significance was inferred based on '*r*' & '*P*' values. Logistic regression analysis adjusted for compounding factors such as age, gender and glycaemic status was performed to assess the role of α_1-AT in the DR group.

Results

Table IA depicts the general characteristics of the control and study groups. The study groups included both males and females. The mean age in years was 50.47±7.99 for controls, 54.22±9.51 for T2DM, 58.82±6.98 for DN and 56.40±9.0 for DR. Comparison of the duration of diabetes with development of complications showed that the mean duration of diabetes was significantly higher for DN (13.10±4.40) compared to DR (9.95±3.83), $P<0.0005$.

Table IB depicts the baseline biochemical parameters of the study groups. The mean glycemic status, and renal function parameters were confirmative for the respective study groups. CRP levels were measured in all the study groups. Diabetes patients with complications had significantly higher levels of CRP (DN 43.40±8.72, DR 23.80±4.68; $P<0.0005$) compared to T2DM (17.90±6.68).

The data on the levels of plasma elastase, α_1-AT, α_2-MG and NE-α_1-AT complex are represented in Table II. There was a significant increase in the NE activity in patient groups with mean levels for T2DM (0.73±0.31), DN (0.87±0.35) and DR (0.76±0.41) compared to control (0.35±0.20). The mean levels of α_1-AT were lowest in DR (8.77±2.85) followed by DN group (26.26±6.16) in comparison with T2DM

Table IA. General characteristics of study groups

Variables	Control (n=60)	T2DM (n=60)	DN (n=60)	DR (n=60)
Age (yr)	50.47±7.99***	54.22±9.51***	58.82±6.98***	56.40±9.00***
Gender (male/female)	35/25	38/22	39/20	36/24
Duration of diabetes (yr)	-	5.10±2.14***	13.10±4.40***	9.95±3.83***

P ***<0.001. Results are presented as mean±SD. SD, standard deviation; T2DM, type 2 diabetes mellitus; DN, diabetic nephropathy; DR, diabetic retinopathy

Table IB. Baseline biochemical parameters of study groups

Variables	Control (n=60)	T2DM (n=60)	DN (n=60)	DR (n=60)
FBG (mg/dl)	91.33±10.47***	165.18±69.72***	191.80±71.92***	186.43±71.82***
PPBG (mg/dl)	-	224.48±84.81***	272.40±83.99***	285.17±86.91***
HbA1c (%)	5.49±0.58*	8.78±2.20*	9.80±2.92*	10.02±2.31*
Blood urea (mg/dl)	22.02±7.8***	26.57±7.56***	59.62±16.46***	30.97±14.13***
Serum creatinine (mg/dl)	0.93±0.25***	1.02±0.12***	2.25±0.50***	1.22±0.85***
Serum CRP (ug/ml)	0	17.90±6.68***	43.40±8.72***	23.80±4.68***

P ***<0.001, *<0.05. Results are presented as mean±SD. FBG, fasting blood glucose; PPBG, post-prandial blood glucose; HbA1c, glycated hemoglobin; CRP, c-reactive protein

Table II. Plasma levels of elastase activity, alpha$_1$-antitrypsin(α_1-AT), alpha$_2$-macroglobulin(α_2-MG) and neutrophil elastase (NE) - alpha1-AT complex in the study groups

Parameters	Control (n=60)	T2DM (n=60)	DN (n=60)	DR (n=60)
Plasma elastase activity (U/ml)	0.35±0.20***	0.73±0.31***	0.87±0.35***	0.76±0.41***
Plasma α_1-AT (mg/dl)	122.95±25.71***	41.13±14.06***	26.26±6.16***	8.77±2.85***
Plasma α_2-MG (mg/dl)	208.87±31.16***	167.29±30.45***	144.66±13.72***	104.67±11.47***
Plasma NE - α_1-AT complex (ng/ml)	215.83±13.61***	98.85±23.85***	129.26±20.40***	153.25±17.11***

P ***<0.001. Results are in mean±SD

(41.13±14.06) and control group (122.95±25.71). The decrease in the levels of α_1-AT was to the extent of 15-fold in DR patients compared to controls, while decrease was four-fold in DN. The levels of α_2-MG were also found lowered in DN (144.66±13.72), DR (104.67±11.47) and T2DM (167.29±30.45) than controls (208.87±31.16). Comparative analysis of the levels of α_1-AT and α_2-MG in DR patients indicated that the decrease was 15-fold for α_1-AT while it was only two-fold for α_2-MG compared to controls. Intergroup comparison of the levels of α_2-MG between DR and DN showed that the fold changes were not as distinct as that of α_1-AT. Plasma levels of NE-α_1-AT complex were 98.85±23.85, 129.26±20.40 and 153.25±17.11, respectively, for DM, DN and DR with controls having a mean value of 215.83±13.61.

The levels of α_1-AT when compared with severity score of DR showed no significant difference (NPDR 8.68±2.96, PDR 8.96±2.70. *P*=0.530). Similarly, gender-wise comparison of levels of α_1-AT also did not reveal any significance (males 8.41±2.71, females 9.37±3.01. *P*=0.247).

As the level of α_1-AT was lowest in the DR group compared to all other study groups, a correlation analysis was therefore carried out between α_1-AT with glycemic status, CRP concentration, NE activity, α_2-MG and NE- α_1-AT complex to associate these variables (Table III). The correlation of α_1-AT with FBG, CRP and NE activity presented negative associations and the association was strong with FBG (*r*=−0.282, *P*=0.029). On the other hand, HbA1c, α_2-MG and NE- α_1-AT complex correlated positively with α_1-AT. Multivariate logistic regression analysis adjusted for age, gender and glycaemic status was carried out to assess the role of α_1-AT in the retinopathy group. The outcome of the analysis presented an odds

Table III. Correlation of plasma α_1-AT with other parameters in diabetic retinopathy group

Plasma α_1-AT	FBG	HbA1c	CRP	Plasma elastase activity	Plasma α_2-MG	Plasma NE - α_1-AT complex
Pearson correlation value (*r*)	−0.282*	0.032	−0.125	−0.126	0.098	0.055
P	0.029	0.807	0.341	0.339	0.458	0.774

*Correlation was significant at 0.05 level

ratio of 0.663, 95 per cent confidence interval 0.0-3.83 which was not significant.

Discussion

Hyperglycaemia-induced endothelial damage has been studied extensively and is considered as one of the critical factors in the development of vascular complications in diabetes[28]. The mechanisms underlying endothelial dysfunction in T2DM are complex and suggestively include oxidative stress and inflammation[29]. Neutrophils respond to inflammation and release proteases leading to tissue damage through proinflammatory mediators. One of the proteases released is NE and under normal physiological conditions, the activity of NE is regulated by α_1-AT and α_2-MG[11]. With this background knowledge, the present study was designed to evaluate the levels of plasma NE, α_1-AT, α_2-MG and NE–α_1-AT complex to understand their roles in the pathophysiology of DN and DR.

The data on the increased levels of NE in T2DM, DN and DR are in agreement with the reported levels in earlier studies, as a consequence of the hyperglycaemia-induced inflammatory response[21]. On the other hand, significantly decreased levels of the α_1-AT and α_2-MG were observed in T2DM, DN and DR patients compared to controls. However, the magnitude of decrease in the levels of α_1-AT and α_2-MG were different among the three patient groups. A noticeable observation was the low levels of α_1-AT in DR group compared to DN and T2DM groups. In other words, the extent of decrease in the levels of α_1-AT was 70 per cent in DR compared to DN and was 80 per cent compared to T2DM. This specifies the selective decrease of α_1-AT in retinopathy patients.

The reasons for such a reduction can be interpreted based on the following perspectives. It has been reported that patients suffering from diabetes exhibit oxidative stress and generate free radicals causing the destruction of anti-proteases. Further, there are studies to indicate that diabetes patients with poor glycaemic control display increased non-enzymatic glycation of circulating α_1-AT leading to inactivation of α_1-AT[30]. The drastic decrease in levels of α_1-AT observed in DR patients than DN patients, therefore, could be due to reasons other than cited above as hyperglycaemia is a common feature both in DN and DR. This observation paved way to consider other molecular mechanisms for the decrease in α_1-AT levels in retinopathy. The possibilities could include decreased expression or mutation in the α_1-AT gene resulting in decreased synthesis or defective/truncated protein formation in retinopathy patients. In support of this hypothesis, a study by Sandström *et al*[22] showed that the frequency of α_1-AT deficiency genotype was 50 per cent higher in individuals with T2DM compared with control subjects. Various research groups working on experimental diabetes in animal models and retinal pericyte cell cultures have demonstrated the potential protective role of α_1-AT on retinal vasculature. The studies also have suggested that early use of α_1-AT therapy may be an effective strategy to prevent or hinder the progression of DR[31,32].

The data on the levels of α_2-MG in the patient groups indicated a significant decrease in its levels compared to controls. There are reports on the increased levels of α_2-MG in T2DM patients with microalbuminuria contrary to the present observation[24]. In a study by Siddiqui *et al*[33], oxidative free radical (H_2O_2) has been shown to be a potent inactivator of α_2-MG. The reduction observed in the levels of α_2-MG therefore could only be attributed to the oxidative damages. Further, the data on the plasma levels of NE–α_1-AT complex indicated a significant decrease in T2DM, DN and DR as compared to controls. Among the patient groups, the DR group showed higher levels and this may be ascribed only to the definitive decreased α_1-AT levels observed in the DR group.

To the best of our knowledge this is the first study with respect to levels of NE and its endogenous inhibitors carried out concurrently in T2DM, DN and DR. This approach has yielded the selective decrease of α_1-AT levels in DR compared to DN. Further, a comparison of the duration of diabetes in DR and DN showed that the mean duration was lower in DR than

DN. This observation iterates the rationale that α_1-AT could be playing a role in the pathophysiology of DR. Therefore, evaluating the levels of α_1-AT in diabetic patients becomes pertinent for comprehensive and effective clinical management of diabetes.

The relatively small sample size of the patient groups, non-inclusion of studies on oxidative stress parameters and molecular genetic studies to evaluate expression and mutation in the α_1-AT gene, however, comprise the study limitations. Considering these in future studies might provide supporting evidence for the observations of this study.

Overall, the homeostatic balance between NE, α_1-AT and α_2-MG is disrupted in T2DM, DN and DR. Modulation of the levels of NE and its inhibitors in individuals with T2DM thus may have relevance in the assessment of complications of diabetes. Highly reduced levels of α_1-AT observed in DR are suggestive of its possible role in the pathophysiology of retinopathy. The outcome of the study also emphasizes α_1-AT as a plausible therapeutic target.

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***Conflicts of Interest*:** None.

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