

## Chitotriosidase activity predicts endothelial dysfunction in type-2 diabetes mellitus

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**Abstract** The enzyme chitotriosidase (ChT) is secreted by activated macrophages and play active role in human immune response. ChT activity is increased in atherosclerosis in association to the extent of the disease. We investigated the relevance of ChT to endothelial functions and insulin resistance in patients with T2DM. Forty newly diagnosed and untreated patients with T2DM (male 17; age  $47.0 \pm 6.2$  years) and 50 healthy volunteers (male 21; age  $50.2 \pm 8.8$  years) were enrolled. Plasma asymmetric dimethyl arginine (ADMA) levels were determined by ELISA. ChT activity was measured by the fluorescence method. Insulin resistance was calculated by the HOMA-IR formula. The patients had higher systolic blood pressures, HOMA-IR, ADMA levels, and ChT activities ( $P < 0.001$  for all) and lower HDL cholesterol levels ( $P = 0.03$ ) than the control group. The ChT activities of the total group were significantly correlated to the age ( $r = 0.031$ ,  $p = 0.003$ ), ADMA ( $r = 0.22$ ,  $p = 0.04$ ), and plasma glucose levels ( $r = 0.27$ ,  $p = 0.01$ ). ChT was the

independent determinant of the plasma ADMA levels ( $r = 0.26$ ,  $p = 0.02$ ). The results of this study show that serum ChT activity is increased in patients with newly diagnosed, untreated, and uncomplicated patients with T2DM. The results also imply that increased ChT activity may be a predictor of endothelial dysfunction.

**Keywords** Type-2 diabetes · Chitotriosidase · ADMA · Endothelial dysfunction · Insulin resistance

### Introduction

Type-2 diabetes mellitus (T2DM) is the leading cause of cardiovascular morbidity and mortality worldwide [1–3]. Poor glucose control, hypertension, and dyslipidemia are the main factors that increase the risk of atherosclerotic disease in T2DM [4]. Whatever the trigger is, chronic inflammation and endothelial dysfunction are the sine qua non in the course of atherosclerosis, from the earliest steps of monocyte recruitment to the complicated phase of plaque rupture [5–8]. Thus, to predict the severity of atherosclerotic process, biomarkers of inflammation and endothelial dysfunction have been a focus of extensive clinical research [9].

The enzyme chitotriosidase (ChT) is one of the most abundant and indicative proteins secreted by activated macrophages [10]. While it has an active role in the immune response [11–13], there is evident data showing a role for it in disease states where inflammatory responses prevail [14–16]. It has been reported that serum ChT activity is significantly increased in patients with established atherosclerosis in relation to the severity of the lesion, suggesting a possible role for ChT as a marker of advanced atherosclerosis [17, 18]. However, the

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mechanism of how ChT activity affects the progression of atherosclerosis, and whether blood level of this enzyme is altered in people at increased risk but without atherosclerotic events are yet unknown.

Endothelial dysfunction and impaired insulin sensitivity play a profound role in increased atherosclerotic progress in diabetes. In this study, we measured ChT serum level in a group of specifically selected T2DM patients, and searched for its relation to the degree of insulin resistance as well as to the level of asymmetric dimethyl arginine (ADMA), a well-established marker of endothelial dysfunction. The results are important since this study is probably the first to display the presence of a novel circulating enzyme activity in a metabolic disorder that is closely associated with atherosclerosis.

## Materials and methods

The participants of the study were selected among the patients who were referred to the check-up center of the Outpatient Clinics of Gulhane School of Medicine, between August 2008 and January 2009 ( $n = 4800$ ). At this period, 184 patients were newly diagnosed as diabetes mellitus. Type-2 diabetes mellitus was defined according to the criteria of the American Diabetes Association [19]. Of these patients, we excluded the ones who already had chronic metabolic disorders including hypertension, dyslipidemia, peripheral or coronary artery disease (history or abnormal ECG findings), thyroid dysfunction, microalbuminuria, retinopathy, or chronic use of any medication ( $n = 132$ ). Also the patients who did not give consent ( $n = 12$ ) were not included. As a result 40 patients (male 17; age  $47.0 \pm 6.2$  years) with newly diagnosed T2DM were enrolled in the study. The control group was selected among the healthy volunteers referred to the same unit at this period. Samples from 50 subjects (male 21; age  $50.2 \pm 8.8$  years) were studied after matching for age, BMI, and gender. Before inclusion, all patients and controls underwent careful physical examination and detailed laboratory investigations to exclude any condition that may interfere with the study parameters. The exclusion criteria implemented to both the patients and the controls were as follows: any evidence of cardiovascular disease, hypertension, dyslipidemia, rheumatic or granulomatous diseases, renal, hepatic or thyroid dysfunction, medications, regular alcohol, or drug consumption.

Standing height and body weight were measured in light indoor clothing without shoes. Body mass index (BMI) was calculated as weight divided by squared height ( $\text{kg}/\text{m}^2$ ). Arterial blood pressure was measured in the two arms by a mercury sphygmomanometer two times in a resting condition in the morning, and mean values were calculated for

diastolic and systolic pressures. The study was approved by the Ethical Committee of Gulhane School of Medicine and all patients and control subjects gave written informed consent.

## Laboratory analyses

The blood samples were collected between 08:00 and 08:30 a.m. after a 12-h fasting. The tubes were promptly centrifuged and plasma was separated and stored at  $-80^\circ\text{C}$ . All samples were run in the same assay. Glucose, total cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride (TG) levels were measured by the enzymatic colorimetric method with Olympus AU 600 auto analyzer using reagents from Olympus Diagnostics (GmbH, Hamburg, Germany). Low-density lipoprotein (LDL)-cholesterol was calculated by Friedewald's formula [20]. The serum basal insulin value was determined by the coated tube method (DPC, Los Angeles, CA, USA). In particular, a HOMA-IR was computed with the formula:  $\text{HOMA-IR} = \text{fasting plasma glucose (mg/dl)} \times \text{immunoreactive insulin (mU/ml)} / 405$  [21].

ChT activity was measured according to the method described previously by Hollak et al. [22]. Briefly,  $5 \mu\text{l}$  of plasma was incubated with  $100 \mu\text{l}$  of  $22 \mu\text{mol/l}$  4-methylumbelliferyl- $\beta$ -D-N'-N''-triacetylchitotriosidase (Sigma M-5639; Sigma-Aldrich ChemieGmbH, Taufkirchen, Germany) in McIlvain's phosphate-citrate buffer;  $\text{pH} = 5.2$ , for 1 h at  $37.0^\circ\text{C}$  in darkness. The reaction was terminated by adding  $120 \mu\text{l}$   $0.5 \text{ mol/l}$   $\text{Na}_2\text{CO}_3$ - $\text{NaHCO}_3$  buffer,  $\text{pH} = 10.7$ . In the quantitative method, the fluorescence of 4-methylumbelliferone was read in a Microfluor 2<sup>®</sup> plate by a fluorimeter (BIO-TEK SynergyHT; Biotek Instruments Inc., Winooski, VT) (excitation 360, emission 450 nm). The ChT activity was expressed as nanomols of substrate hydrolyzed per milliliter per hour ( $\text{nmol}/\text{ml}/\text{h}$ ).

Plasma ADMA level was determined by ELISA (ADMA direct ELISA kit, Immundiagnostik AG, Bensheim, Germany) [detection limit of ADMA assay =  $0.05 \mu\text{mol/l}$ ]. Measurements were carried out using ELISA plate reader Bio-Tek Synergy HT (Biotek Instruments Inc., Winooski, VT, USA).

## Statistical analyses

Results were reported as mean  $\pm$  SD. The Levene's test was used to evaluate the distribution characteristics of variables. Differences between T2DM and control groups were tested for significance by *t* test, Mann-Whitney *U* test, or Chi-square test, where necessary. The relationship between variables was analyzed by Pearson's correlation.

Differences and correlations were considered significant at  $P < 0.05$ .

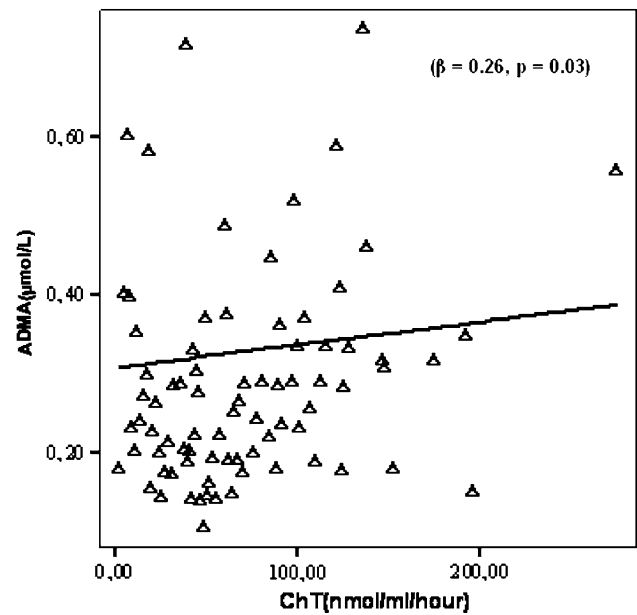
**Results**

The characteristics of the patients and the controls are given in Table 1. Both groups were similar in age, gender, waist circumference (WC), and BMI distributions. When compared to the control group, the patients with T2DM had significantly lower HDL cholesterol levels ( $P = 0.03$ ) and higher systolic blood pressures (SBP), HOMA-IR, ADMA, and ChT activities ( $P < 0.001$ , for all). HDL cholesterol levels of the patients were lower than the controls ( $P = 0.03$ ). After the correction for age, the ChT activities in patients were still significantly higher when compared to those of the controls ( $84.66 \pm 44.28$  vs.  $53.53 \pm 44.12$  nmol/ml/h;  $P = 0.001$ ).

A correlation analysis was performed to test the relation of ChT to the parameters tested. It was found that the ChT activity in the total group was significantly correlated to the age ( $r = 0.31$ ,  $P = 0.003$ ), circulating ADMA concentration ( $r = 0.22$ ,  $P = 0.04$ ) and plasma glucose level ( $r = 0.27$ ,  $P = 0.01$ ). In the multivariate linear regression analysis, when age, BMI, systolic and diastolic blood pressure, total cholesterol, HDL cholesterol, and LDL cholesterol were added to the model, ChT was the independent determinant of the plasma ADMA level ( $\beta = 0.26$ ,  $P = 0.03$ ) (Fig. 1).

**Discussion**

The chitinase enzyme family hydrolyzes chitin, a structural component found in the cell walls of many living species



**Fig. 1** The regression curve of the chitotriosidase activity and ADMA levels

such as the fungi, nematodes, protozoan parasites, and insects [23–25]. ChT is the first discovered human chitinase and one of the most quantitative proteins secreted by activated macrophages. However, the biological function of ChT in humans is not clear. It is considered as a marker of macrophage activity and an important player during the immunological response [10]. ChT might have a role in a tissue-remodeling processes [26] or chemotaxis [27, 28]. ChT activity is increased up to 55-fold in extracts of atherosclerotic tissue, demonstrating a clear association between ChT expression and lipid-laden macrophages in the atherosclerotic vessel wall [17, 18, 29]. Despite the evidence about the role of ChT in the atherosclerosis

**Table 1** The characteristics of the patients with T2DM and healthy controls

	Patients (n = 40)	Healthy controls (n = 50)	P
Age (years)	50.2 ± 8.8	47.0 ± 6.2	0.06
Gender (M/F)	17/23	21/29	0.56
WC (cm)	97.92 ± 8.6	94.60 ± 8.6	0.08
BMI (kg/m <sup>2</sup> )	29.5 ± 3.8	28.1 ± 3.0	0.05
SBP (mmHg)	127.8 ± 9.9	117.6 ± 6.9	<0.001
DBP (mmHg)	79.6 ± 7.4	77.2 ± 6.1	0.09
FBG (mg/dl)	153.8 ± 55.3	90.9 ± 6.6	<0.001
Total cholesterol (mg/dl)	215.3 ± 75.0	219.4 ± 40.8	0.73
Triglyceride (mg/dl)	166.5 ± 68.1	144.6 ± 87.4	0.20
HDL cholesterol (mg/dl)	46.3 ± 12.0	52.2 ± 12.7	0.03
LDL cholesterol (mg/dl)	127.4 ± 36.8	137.4 ± 36.8	0.28
HOMA-IR	4.56 ± 3.37	1.96 ± 0.96	<0.001
ADMA (µmol/l)	0.36 ± 0.11	0.24 ± 0.1	<0.001
ChT (nmol/ml/h)	88.1 ± 54.9	50.7 ± 37.11	<0.001

Data given mean ± SD. Student's *t* test

WC waist circumference, BMI body mass index, SBP systolic blood pressure, DBP diastolic blood pressure, FBG fasting blood glucose, HOMA-IR homeostasis model assessment index, ADMA asymmetric dimethyl arginine, ChT chitotriosidase

development, there has been no data so far about the ChT activity in T2DM, a major cause of accelerated atherosclerosis. Moreover, there has been no report to date, about any relationship between ChT activity and endothelial functions or insulin resistance.

The results of this study show that plasma ChT activities are significantly elevated in patients with newly diagnosed, treatment naïve, uncomplicated T2DM. Also, the elevation in ChT activity is associated with the age, plasma glucose, and ADMA levels. The age-dependent increase in ChT levels has been reported previously [30, 31]. This was explained by the lifelong accumulation of lipid-laden macrophages during the gradual progression of atherosclerosis [10]. However, the relationship of ChT activity to the plasma glucose or ADMA levels is a new finding. The rise in the ChT activity is found to be an independent determinant of plasma ADMA level as well. It is clearly known that ADMA is the major inhibitor of nitric oxide biosynthesis and its increased concentration is a significant determinant of endothelial dysfunction [32, 33]. Significantly elevated ADMA level and accompanying endothelial dysfunction have been reported in a number of chronic metabolic disorders including T2DM, hypertension, dyslipidemia, and chronic kidney disease [33–36]. Because of the established role of ADMA levels in atherosclerosis and endothelial dysfunction, it is used as an index parameter in this study. Thus, the association between the ChT activity and the circulating ADMA concentration suggest that ChT activity may modulate endothelial functions in patients with T2DM.

ChT measurement is easy, reproducible, reliable, and cost effective. As it does not necessitate any kit, it is always possible to make routine ChT measurements on daily basis for each patient. Also, ChT is a very stable enzyme which can allow direct comparison of plasma and serum samples that have been stored under widely different conditions. Multiple cycles of freeze drying had no effect on chitotriosidase activity in plasma or serum [37]. However, whether measuring ChT activity provides useful information about the atherosclerotic risk in T2DM or other chronic metabolic diseases is not clear yet. Since atherosclerotic risk biomarkers should provide independent information on the cardiovascular risk [9], ChT activity should be assessed in other disease states that are associated with increased cardiovascular morbidity and mortality. Currently, ChT activity is in use as a diagnostic tool for monitoring the efficacy of therapy in Gaucher's disease [22] or  $\beta$ -glucocerebrosidase deficiency [38]. ChT gene expression is increased in the Kupffer cells of the patients with non-alcoholic fatty liver disease (NAFLD) [16], and such an increase in ChT activity was reported to have a potential role in the pathogenesis of NAFLD [15]. On the other hand, the ChT activity was

found unchanged in patients with dyslipidemia and it was not found to have a relation with plasma lipid levels [3]. There is no data about other metabolic diseases including hypertension, metabolic syndrome, or chronic kidney disease which are, like T2DM, among the most important causes of atherosclerosis. The present data is the first performed in patients with T2DM, and these preliminary results need to be validated in several other case control studies as well as prospective trials designed to test whether blood ChT activity is affected from glucose lowering interventions.

This study may have several limitations. As of the case-control nature of the design, the association between the ChT activity and the ADMA levels do not necessarily indicate causality. Also, the correlation between ADMA and ChT is weak and may not be clinically relevant. We think that the lack of normal range for chitotriosidase and the relatively small number of the study group are important factors that prevent much stronger association. It is also possible that, low ChT activity levels due to the inherited defects of the ChT gene might have confounded the results. Decreased ChT activity is common in different ethnic groups [10], while 3–5% of the general population has no ChT activity due to the presence of a null allele in the ChT gene [39]. However, in order to prevent perplexity, subjects with the ChT activity levels below 10 nmol/ml/h were omitted. Finally, due to the strict selection criteria (i.e., untreated diabetics with no other diseases or drug use), the study group may not perfectly represent the diabetic population.

In conclusion, the results of this study show that serum ChT activity is increased in patients with newly diagnosed, untreated, and uncomplicated patients with T2DM. The results also imply that increased ChT activity may be a predictor of endothelial dysfunction. However, it should be noted that this is only a preliminary report to search for the relevance of chitotriosidase enzyme activity in T2DM. Future designs to apprise the relation of chitotriosidase to clinical outcome parameters will probably give more critical information about the clinical relevance of ChT activity in endothelial dysfunction.

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