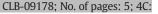
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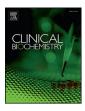
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## Association of chitotriosidase enzyme activity and genotype with the risk of nephropathy in type 2 diabetes

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## A R T I C L E I N F O

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## ABSTRACT

**Objective.:** The immune-inflammatory system has been implicated in the pathogenesis of diabetic nephropathy; however, many of the mechanisms involved remain unclear. Chitotriosidase enzyme is an active human chitinase and a major protein product of activated macrophages. Although playing an important role in innate and acquired immunity, chitotriosidase involvement in the development of diabetic nephropathy is unknown.

**Design and methods.:** Chitotriosidase enzyme activity and the presence of the functional 24-bp duplication mutation of the chitotriosidase gene (*CHIT1*) were assessed in 262 Egyptian type 2 diabetic patients with and without nephropathy and 90 non-diabetic controls. In diabetic patients, multiple linear regression models were adapted to assess the association of chitotriosidase activity with two important measures of renal disease progression: urinary albumin/creatinine ratio and eGFR, while the association of the *CHIT1* genotype with the incidence of nephropathy was evaluated by multiple logistic regression.

**Results:** In diabetic patients, chitotriosidase enzyme activity showed a statistically significant elevation as compared to controls and correlated positively with the progression of nephropathy. A significant association of chitotriosidase activity with both urinary albumin/creatinine ratio and eGFR was detected after adjusting for age, gender, duration of diabetes, body mass index, hypertension status, total cholesterol, triglycerides and HbA1c levels, P < 0.001. We also identified a protective association between the *CHIT1* mutated genotype and diabetic nephropathy after adjusting for the same confounders (odds ratio: 0.517, 95% CI: 0.289–0.924, P = 0.026). **Conclusions:** This study demonstrates for the first time that the immunomodulatory effects of chitotriosidase

enzyme could be implicated in the development of nephropathy in type 2 diabetes.

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## 1. Introduction

Diabetic nephropathy is an important microvascular complication of diabetes mellitus and the most common cause of end stage renal disease (ESRD) [1]. About 20 to 40% of patients with type 1 or type 2 diabetes develop evidence of nephropathy and for type 2 less than 40% of those will progress to ESRD [2]. The development of diabetic nephropathy is a very complex process affected by age, ethnicity, duration of diabetes, glycemic control status, associated hypertension, life style factors and multiple genetic predispositions [3,4]. Many studies including in-vitro cellular experiments, animal model experiments and epidemiological studies linked inflammation to pathogenic mechanisms in diabetic

nephropathy [5]. Macrophage migration and recruitment, especially M1 subpopulations, directly contribute to renal injury in diabetes, perhaps by altering podocyte integrity [6] and directly correlate with the degree of renal fibrosis [7]. Furthermore, several immunomodulatory molecules such as chemokines (CCL2, CX3CL1 and CCL5), adhesion molecules (intercellular adhesion molecule 1, vascular cell adhesion protein 1, E-selectin and  $\alpha$ -actinin 4), and cytokines (IL-1, IL-6, IL-18 and tumor necrosis factor- $\alpha$ ) have all been implicated in the pathogenesis of diabetic nephropathy [5].

Chitotriosidase enzyme is a fully active human chitinase that was initially discovered to be markedly elevated in the plasma of patients with Gaucher's disease, an inflammatory-based lysosomal storage disorder [8]. Later, its elevation was detected in other lysosomal and non-lysosomal inflammatory disorders [9–12]. Chitotriosidase is both an endo- and exo-chitinase splitting chitin molecules into smaller polysaccharide moieties and also to its basic monosaccharide N-acetylglucosamine [13]. The absence of its substrate chitin in mammals and the exclusive expression in immune cells, mainly activated macrophages, elucidated its involvement in the activation

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Abbreviations: BMI, body mass index; CKD-EPI equation, chronic kidney disease epidemiology collaboration equation; ESRD, end stage renal disease; eGFR, estimated glomerular filtration rate; TBRI, Theodor Bilharz Research Institute.

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of the innate and acquired immune systems, especially against chitin coated pathogens [13,14]. Many immune enhancing molecules such as tumor necrosis factor- $\alpha$ , interferon- $\gamma$  and lipopolysaccharide promote chitotriosidase expression in activated macrophages [15], while chitotriosidase was shown to stimulate IL8, CCL2, CCL5 and eotaxin and to increase the migratory capacity of eosinophils, T lymphocytes and macrophages [16]. Chitotriosidase also stimulates transforming growth factor- $\beta$ 1 receptor expression and signaling suggesting a role in enhancing the response to organ injury and repair. For example, bleomycin-induced pulmonary fibrosis was significantly reduced in chitotriosidase null mice and significantly enhanced in chitotriosidase over-expressing transgenic mice [17].

The 24-bp duplication mutation (rs3831317) at exon ten of the chitotriosidase gene (*CHIT1*), resulting in aberrant splicing and deletion of 87 nucleotides, is the main cause of complete enzyme deficiency in about 6% of Caucasians when homozygously mutated. It also reduces the chitotriosidase enzyme expression in heterozygous individuals [18]. In this cross-sectional study we aimed to investigate the potential role of chitotriosidase enzyme and its functional mutation (24-bp duplication) in the development and progression of nephropathy in a cohort of Egyptian type 2 diabetic patients.

### 2. Patients and methods

## 2.1. Patients

For the current study, 262 Egyptian type 2 diabetic patients (48.6  $\pm$  5 y, 42.3% males) for a minimum duration of ten years, and 90 nondiabetic controls (43.5  $\pm$  4.7 y, 38.9% males) were recruited from patients presenting to the internal medicine and nephrology outpatient clinics at Theodor Bilharz research institute (TBRI) and blood donors visiting the blood bank at TBRI, respectively. Enrolment of patients extended over the period from October 2013 to December 2014. Diabetic patients were further divided into patients without diabetic nephropathy evidenced by urinary albumin excretion <3.4 mg/mmol creatinine (n = 84, 47.4  $\pm$  5.2 y) and patients with diabetic nephropathy (n = 178, 49.3  $\pm$  4.7 y), presenting with either microalbuminuria (3.4–34 mg/mmol creatinine, n = 88, 47.8  $\pm$  4.1 y) or macroalbuminuria (>34 mg/mmol creatinine, n = 90, 51  $\pm$  4.6 y). Body mass index (BMI) in control subjects was matching type 2 diabetic patients.

Demographic and clinical data were recorded including age, gender, BMI, duration of diabetes and history of previous episodes of myocardial infarction or stroke. Associated hypertension was determined based on blood pressure levels over 140/90 mm Hg or the intake of antihypertensive medications, while the presence of other microvascular complications such as retinopathy and/or neuropathy was based on standard fundoscopy and neurological examinations, respectively. Routine laboratory investigations including fasting plasma glucose, urea, creatinine, urinary albumin creatinine ratio, total bilirubin, alanine transaminase, total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol were assayed by Synchron CX5 (Beckman Coulter, Brea, California, USA). HbA1c was assayed by the ion-exchange resin separation method (Human Diagnostics, Wiesbaden, Germany). Estimated GFR (eGFR) calculation was based on the chronic kidney disease epidemiology collaboration (CKD-EPI) equation. [GFR = 141  $\times$  min(Scr/ĸ, 1)^{\alpha}  $\times$  $max(Scr/\kappa, 1)^{-1209} \times 0.993^{Age} \times 1018$  (if female) × 1159 (if black)], where Scr = serum creatinine,  $\kappa$  = 0.7 if female and 0.9 if male,  $\alpha = -0.329$  if female and -0.411 if male, min = the minimum of  $Scr/\kappa$  or 1 and max = the maximum of  $Scr/\kappa$  or 1 [19].

Diabetic patients with hepatic disease, heart failure, neurological or other endocrinological diseases, cancers or any acute or chronic infections were excluded from the study. A written informed consent was obtained from all diabetic patients and non-diabetic controls. The study protocol was in accordance with the declaration of Helsinki 1975 and as modified in 2012, and approved by the institutional review board of TBRI before the start of enrolling participants (Reference number: FWA 00010609).

## 2.2. Chitotriosidase enzyme activity

Plasma chitotriosidase activity was measured as described previously [20]. Ten microliters of plasma were mixed with 100  $\mu$ L of 0.022 mmol/l 4-methylumbelliferyl- $\beta$ -D-N,N',N"-triacetylchitotrioside (Sigma) in citrate/phosphate buffer, 0.1/0.2 mol/L, pH 5.2 and incubated at 37 °C for 15 min. The reaction was stopped with 2 mL of 0.5 mol/L carbonate/bicarbonate buffer, pH 10.7. Fluorescence was measured by FP 6200 (Jasco analytical instruments, Tokyo, Japan) at excitation wavelength 365 nm and emission wavelength 448 nm. Enzyme activities were calculated based on a calibration curve of 4-methylumbelliferone and expressed as nmol of enzyme product formed/mL plasma/h.

### 2.3. Chitotriosidase genotype

DNA was extracted from EDTA blood using the GeneJET DNA purification kit (Thermo Scientific, MA, USA) according to the manufacturer's protocol. The detection of the 24-bp duplication mutation within exon ten of CHIT1 gene was performed as previously described [18]using the primers 5'-AGCTATCTGAAGCAGAAG-3' and 5'-GGAGAAGCCGGC AAAGTC-3' as forward and reverse primers, respectively. PCR protocol constituted of initial denaturation at 95 °C for 3 min followed by 35 cycles of: denaturation at 95 °C for 30 s, annealing at 56 °C for 60 s, extension at 72 °C for 30 s and final extension at 72 °C for 7 min. Detection of PCR amplification products was performed using either 4% agarose or 10% polyacrylamide gel electrophoresis, ethidium bromide as a staining dye and ultraviolet transillumination; revealing the formation of a 75-bp band in the wild-type, a 99-bp band in the homozygously mutated and both in the heterozygous. Genotype assessment was repeated in a random sample consisting of 25% of all subjects.

### 2.4. Statistical analysis

Comparisons of quantitative data were analyzed using Mann-Whitney U-test or Kruskal–Wallis test for differences between medians. Pearson chi-square ( $\chi^2$ ) test was used to test categorical variables among groups and also to test the Hardy-Weinberg equilibrium for the mutation genotype and allele frequencies among controls and patients. Association of chitotriosidase enzyme activities with the continuous variables urinary albumin/creatinine ratio and estimated GFR was performed through multiple linear regression after adjusting for age, gender, BMI, duration of diabetes, hypertension status, total cholesterol, triglycerides and HbA1c levels. The 24-bp duplication mutation was also associated with the dichotomous variable diabetic nephropathy through multiple logistic regression adjusted for the same confounders. Associations were expressed as regression coefficients with standard errors for linear regressions and as odds ratios with 95% confidence intervals (CI) for logistic regressions. A 2-sided P value < 0.05 was considered significant. Sample size calculation was based on preliminary data obtained from a pilot study performed in 50 type 2 Egyptian diabetic patients, whose data are not included in the current study. Statistical analysis was performed by the WINPEPI statistical software package, version 11.43 [21].

## 3. Results

The study sample consisted of 262 Egyptian type 2 diabetic patients and 90 non-diabetic controls. Demographic, clinical and biochemical data of study participants are summarized in Table 1. Chitotriosidase enzyme activities (median; 25th–75th percentiles) were significantly elevated in diabetic patients (53; 30–96 nmol/mL plasma/h) as compared to non-diabetic controls (37; 31–46 nmol/mL plasma/h), P <0.001. Furthermore, there was a significant increase in chitotriosidase

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