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Research Article
Is Urinary Calprotectin a Useful Tool to Detect the Therapeutic Control of Type II Diabetes?

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Abstract

Background and Objective: The use of plasma calprotectin; a protein mainly expressed in neutrophils; as a diagnostic and/or prognostic marker in type 2 diabetic patients is still controversial. Being a non-invasive test, this study was designed to detect if urinary calprotectin could be used to differentiate between treated-controlled and treated-uncontrolled type II diabetic patients. **Materials and Methods:** Based on levels of fasting blood sugar (FBS), postprandial blood sugar (PPBS) and glycated hemoglobin (HbA1c), 200 adult diabetic patients on regular anti-diabetic treatment were equally divided into four groups: controlled non-obese (controlled diabetics with no obesity) and controlled obese (controlled diabetics with obesity), uncontrolled non-obese (uncontrolled diabetics with no obesity) and uncontrolled obese (uncontrolled diabetics with obesity) in addition to a healthy control group (non-diabetic subjects with no obesity). The exclusion criteria included renal diseases, use of medications other than anti-diabetics, infection, use of antibiotics two weeks before the study, major comorbidities, pregnancy and lactation. Urine samples were collected for assay of calprotectin. **Results:** The level of urinary calprotectin did not differ significantly in all groups. Also Pearson correlation and the regression analysis showed non-significant correlations between urine calprotectin and FBS, PPBS or HbA1c in all groups. **Conclusion:** Urinary calprotectin levels did not differ significantly between controlled and uncontrolled patients with type 2 diabetes whether obese or not indicating that it cannot be used to differentiate between treated-controlled and treated-uncontrolled patients with type II diabetes.

Key words: Urinary calprotectin, correlation, fasting blood sugar, postprandial blood sugar, glycated hemoglobin, controlled non-obese

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Identification of biomarkers of a certain disease in apparently-healthy people detects individuals at risk and undiagnosed cases and in patients it predicts therapeutic response and long-term outcome. Biomarkers could be molecular, functional or anatomical signs[1]. Calprotectin is a protein expressed mainly in myeloid cells encompassing neutrophils and monocytes and in endothelial cells. It plays an important role in pathophysiology of different inflammatory diseases and hence its inhibition is a promising potential therapy for inflammatory disorders such as rheumatoid arthritis[2]. It is released from the myeloid cells into body fluids and tissues in inflammatory conditions[3]. Faecal calprotectin is a valuable biomarker for diagnosis and monitoring of the inflammatory bowel disease[4] and colorectal cancer[5]. The first-degree relatives of ulcerative colitis patients have been shown to have higher faecal calprotectin level indicating that they may have a subclinical or minimal intestinal inflammation[6]. Assay of calprotectin in urine and serum by a commercially available ELISA kit rather than by a western blot will support its wide use as a diagnostic and/or prognostic test because ELISA testing is faster, quantitative and easily conducted in clinical laboratories[7]. This will be useful for a widely prevalent disease like type II diabetes.

Most patients with type 2 diabetes are obese[8]. However a large number of patients with type 2 diabetes are non-obese. Compared with obese type 2 diabetics, the non-obese ones have less insulin resistance but a similar risk of cardiovascular disease, higher amount of abdominal and total fat masses and may need insulin therapy earlier[9]. In normal individuals, plasma calprotectin was higher in obese compared to unobese and it decreased after loss of weight[10]. In type 2 diabetics, plasma calprotectin did not differ between obese and unobese and it could be a marker of obesity in those without type 2 diabetes[11]. In contrast, the levels of calprotectin in serum and urine were detected to be related to chronic mild inflammation and insulin resistance beyond obesity[12]. In addition, in type 2 diabetic patients higher levels of plasma calprotectin was associated with obesity, metabolic syndrome, autonomic neuropathy and cardiovascular complications[13]. Serum calprotectin level was found to be higher in diabetics compared to healthy controls and also in diabetics with neuropathy compared with those without[14]. Urinary calprotectin level was found to be an early marker of diseases associated with inflammation and increased neutrophil activity[15]. Elevation of urinary calprotectin could be a promising biomarker for detecting urothelial carcinoma of the bladder[16]. The urothelial (transitional cell) carcinoma is the most common type of bladder cancer and its median age of occurrence is around 69 years but it can occur at any age. In patients less than 40, it is usually of low grade but these patients are managed in the same way like the older ones[17]. Increased levels of urinary biomarkers in type 2 diabetes could be due to renal damage, oxidative stress, chronic minimal inflammation and endothelial damage[18]. Inflammation participates in pathogenesis of type II diabetes, thus immunomodulator therapy may be a new strategy of treatment which decreases both blood sugar and diabetic complications[19].

Consequently, based on and the elevation of serum calprotectin level in diabetics[14], the increase of urinary calprotectin level in inflammatory diseases[15] and the anticipated role of inflammation in pathogenesis of diabetes[19], the current study was carried on type II diabetic patients taking their regular anti-diabetic medications to detect if assay of urine calprotectin could be an easy and non-invasive way to differentiate between controlled and uncontrolled patients.

MATERIALS AND METHODS

Study design and subject recruitment: This study was approved by the Research Ethics Committee of Ministry of Health (No.: A00518) and was performed in accordance with the Helsinki Declaration. Informed consent had been obtained from the participants involved. Adult patients (36-64 years old) with type 2 DM were recruited from the primary health care centers in Jeddah, SA between December, 2017 and July, 2018. The exclusion criteria included: (1) Past or present history of renal diseases, (2) Use of medications other than the anti-diabetics, (3) Infection or use of anti-biotics in the last two weeks before the study, (4) Presence of major comorbidity such as hypertension, malignancy or liver failure and (5) Pregnancy and lactation. Two hundred diabetic patients were equally divided into four groups: Controlled non-obese (controlled diabetics with no obesity) and controlled obese (controlled diabetics with obesity), uncontrolled non-obese (uncontrolled diabetics with no obesity) and uncontrolled obese (uncontrolled diabetics with obesity) in addition to a healthy control group (non-diabetic subjects with no obesity). According to WHO, subjects with body mass index (BMI) $\geq$30 kg m^{-2} were considered obese[20]. The patients were on their regular anti-diabetic medications. According to the American Diabetes Association Criteria[21], the diabetic patient was considered controlled if person had a fasting blood sugar (before a meal) equal to 70-130 mg dL^{-1},

a 2 h-postprandial blood sugar (2 h after starting a meal) less than 180 and a glycated hemoglobin (HbA1c) less than 7%. Written informed consents were obtained from all participants.

Assay of blood sugar levels and glycated hemoglobin (HbA1C): Blood samples, before and 2 h after meals were collected. The levels of blood sugars were measured by the glucose oxidase method[22] and the level of HbA1c was measured as previously described[23] using the commercially available kits (Crystal Chem., IL, USA).

Assay of urinary calprotectin: Morning mid-stream urine samples were collected in sterile containers. Each sample was centrifuged to remove debris (1500xg at 4°C for 15 min) and then the supernatant was transferred to a fresh tube. The supernatants were stored at -80°C. Before measuring, samples were brought to room temperature and mixed gently[7]. Quantification of calprotectin in urine was done using an ELISA kit according to the manufacturer's procedure (Hycult Biotech Inc., PA, USA). Briefly, calprotectin in the sample binds to the immobilized anti-calprotectin anti-body in the wells during the first incubation step. After washing, a detection anti-calprotectin antibody was added in a next incubation step. Then a peroxidase labeled streptavidin conjugate was added. After washing, the substrate for peroxidase was added. After incubation, the stop solution was added and absorbance was read immediately at 450 nm. The concentration was obtained by using the standard curve[12].

Statistical analysis: The SPSS version 22 was used. Continuous data was given as mean values±SEM. Normality of data was checked by Shapiro-Wilk test, skewness, kurtosis and graphs. The one-way analysis of variance (ANOVA) with Bonferroni test was used for multiple comparisons. The Pearson correlation analysis was done to detect the correlation between urine calprotectin and FBS, PPBS and HbA1c in all groups. The significance level was set at $p<0.05$.

RESULTS

Level of urinary calprotectin: The Table 1 showed the levels of FBS, PPBs, HbA1c and urinary calprotectin in the four diabetic groups (controlled non-obese, controlled obese, uncontrolled non-obese and uncontrolled obese) and in the normal control group. It was found that the level of urinary calprotectin did not differ significantly in the four diabetic groups compared with the normal control group and in-between each other.

Correlation between levels urinary calprotectin and diabetic parameters: The Pearson correlation analysis showed non-significant correlations between the level of urinary calprotectin and the levels of FBS, PPBS or HbA1c in both diabetic-controlled and diabetic-uncontrolled groups ($p = 0.49$, 0.99 and 0.12, respectively). Also in all groups, the regression analysis showed non-significant relationships between urine calprotectin and FBS, PPBS or HbA1c (Fig. 1). The data in Table 1 showed that values of the regression coefficient (R^2) between urinary calprotectin and FBS, PPBS or HbA1c (0.002, 1.023E-8 and 0.01 respectively) were very low indicating non-significant correlations.

DISCUSSION

This study investigated that the levels of urinary calprotectin were similar in controlled and uncontrolled diabetic patients whether obese or not indicating that it cannot be used as a parameter to detect effect of treatments in controlling type II diabetic patients whether obese or not. However, large blinded clinical studies are recommended to verify these results. Calprotectin is as a component of the innate immune system. It promotes recruitment and infiltration of polymorphonuclear cells and macrophages into sites of the inflammation[24]. The results of the few studies addressing its correlation with diabetes mellitus are controversial. It was found that in obese individuals the adipose tissue dysfunction leads to chronic inflammatory-

Table 1: Levels of UC, FBS, PPBS and HbA1c in controlled and uncontrolled diabetic groups

Groups	UC (ng mL^{-1})	FBS (mg dL^{-1})	PPBS (mg dL^{-1})	HBA1c (%)
NC	8.39±0.61	96.52±2.02	128.41±1.22	5.47±0.11
CNO	7.84±0.47	103.66±2.34	136.63±3.59	6.02±0.10
CO	9.01±0.49	107.60±2.60	144.00±4.55	5.73±0.11
UCNO	6.98±0.48	175.96±3.47*	293.80±9.97*	10.64±0.23*
UCO	8.87±0.57	182.26±3.43*	304.24±10.01*	11.31±0.27*
R^2 values between UC and DP	-	0.002	1.023E-8	0.01

Data are expressed as Mean±SEM, UC: Urinary calprotectin, FBS: Fasting blood sugar, PPFBS: Post-prandial blood sugar, HbA1c: Glycated hemoglobin, NC: Normal control, CNO: Controlled non-obese, CO: Controlled obese, UCNO: Uncontrolled non-obese, UCO: Uncontrolled obese, R^2: Regression coefficient, DP: Diabetic parameter, *$p<0.05$ vs. NC, CNO and CO

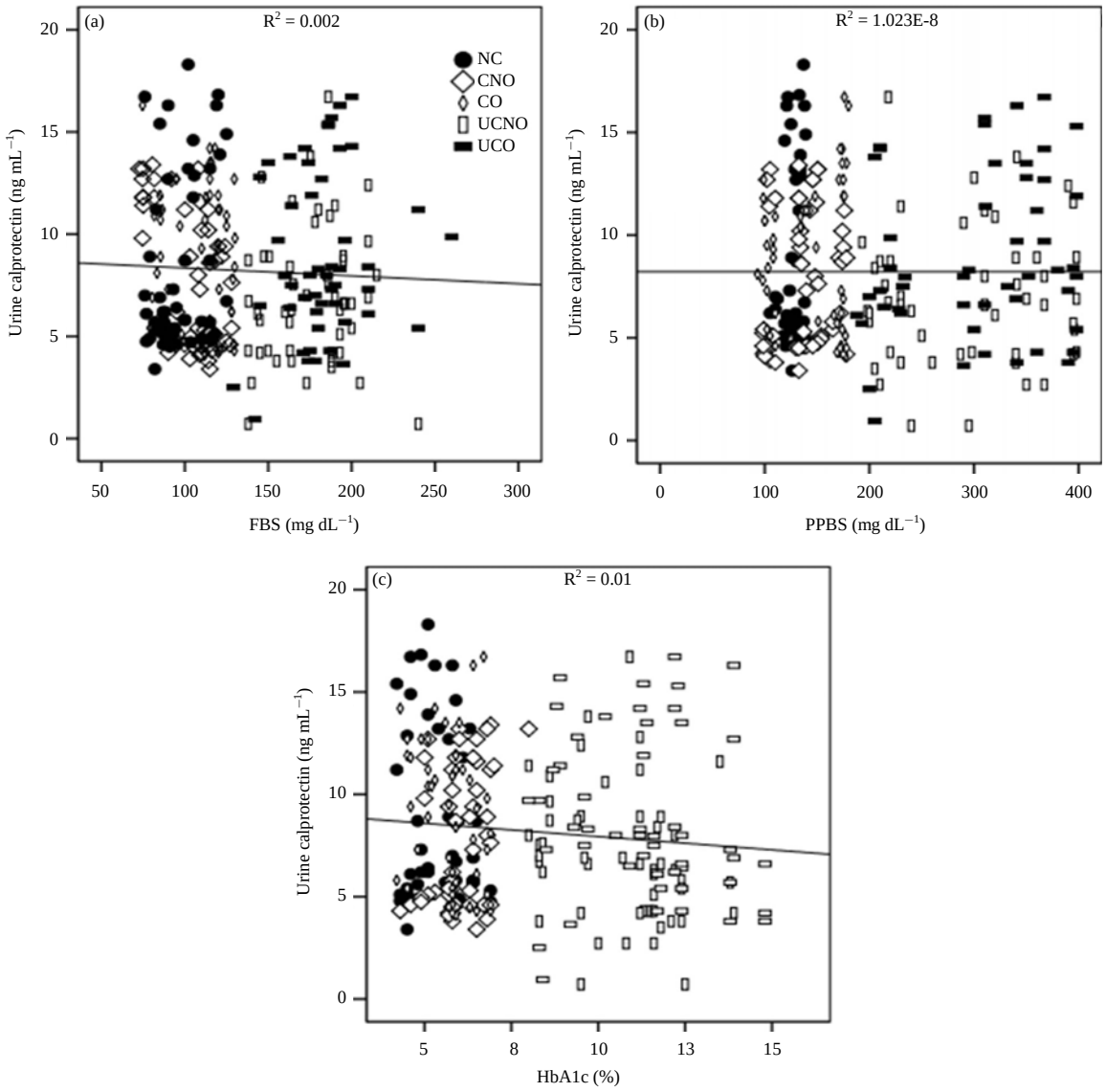

Fig. 1(a-c): Regression analysis between UC and (a) FBS, (b) PPBS and (c) HbA1c in controlled and uncontrolled diabetic groups
UC: Urinary calprotectin, FBS: Fasting blood sugar, PPFBS: Post-prandial blood sugar, HbA1c: Glycated hemoglobin, NC: Normal control, CNO: Controlled non-obese, CO: Controlled obese, UCNO: Uncontrolled non-obese, UCO: Uncontrolled obese. R^2: Regression coefficient, data are expressed as individual observations in each group (n = 50)

related complications[25], thus increased circulating and urine calprotectin could be present in persons with obesity and/or insulin resistance due to release of calprotectin from endothelial cells and macrophages in adipose tissue in addition to circulating polymorphonuclear cells[10]. An association was revealed between serum calprotectin and microalbuminuria which is a marker of cardiovascular disease in type 2 diabetic patients[26]. Stimulation of the advanced glycation end products (RAGE) receptor revealed new aspects into diabetes and its complications[27]. RAGE is a multi-ligand receptor that can be stimulated by calprotectin[28]. A positive correlation was detected between serum calprotectin and HbA1c, which is the marker of long-term increase of blood glucose. This suggested that concentrations of glucose or glycation products could affect regulation of high calprotectin levels in case of diabetic complications[14].

Calprotectin can activate the nuclear factor-kappa B (NF-κB) through activation of toll like receptors 4 (TLR4). NF-κB is a transcription factor that can increase expression of several inflammatory mediators and its activation has been found

associated with diabetic neuropathy[29]. Inflammatory cytokines are also possible important factors in the occurrence of diabetes-induced renal damage[30]. Thus the increased levels of calprotectin in plasma and urine may associate insulin resistance, type 2 DM and diabetic complications such as renal failure and neuropathy. Moreover, weight loss significantly decreased circulating levels of calprotectins[12]. It is known that weight reduction decreases expression of proinflammatory factors while increases expression of anti-inflammatory factors[31]. It was found that treatment with pioglitazone for 3 months decreased serum calprotectin levels in type 2 diabetic obese patients without altering the body mass index[32]. Although, the current study showed a slightly significant regression analysis between urine calprotectin and FBS but urine calprotectin cannot be used as a marker for monitoring treatment of diabetes mellitus due to non-significant relationship between it and HbA1c which is the marker of long-term control of diabetes. An advantage of the study that it used ELISA kit in detecting quantitative levels of calprotectin adding to the attempts of development of a quick assay of it in urine and serum[7].

CONCLUSION

Urinary calprotectin levels did not differ significantly between controlled and uncontrolled diabetic patients whether obese or non-obese indicating that it cannot be used as a marker to differentiate between treated-controlled and treated-uncontrolled patients with type II diabetes.

SIGNIFICANCE STATEMENT

This study discovered that urinary calprotectin cannot be used as a parameter to detect effect of treatments in controlling type II diabetic patients whether obese or not. Therefore, it generates a crucial hypothesis regarding use of urinary calprotectin as a parameter for controlling diabetes. This study will help researchers and physicians to uncover the critical areas of non-invasive markers of controlling diabetes which is an area of a great interest.

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