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Serum CCN3 levels are increased in type 2 diabetes mellitus and associated with obesity, insulin resistance and inflammation

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ABSTRACT

Backgroud: CCN3 is a novel adipokine and has emerged as a potential metabolic regulator. However, information regarding the role of CCN3 in type 2 diabetes mellitus (T2DM) remains unclear. This study measured for the first time serum CCN3 levels in T2DM and explored the correlations between its serum levels and various metabolic parameters in humans.

Methods: A total of 219 newly diagnosed T2DM (nT2DM) patients and 205 healthy control subjects, matched for age and sex ratio, were enrolled. Circulating CCN3 and TNF-α, IL-6 and MCP-1 were measured by ELISA. The anthropometric assessment and biochemical evaluation were done in all subjects. OGTT were performed in 34 healthy individuals to investigate the association of CCN3 with glucose.

Results: Serum CCN3 levels were significantly higher in nT2DM patients compared to those of the healthy controls (6.71[4.88, 8.56] *vs.* 4.51[3.55, 5.99] ng/ml, $P < 0.01$). Serum CCN3 positively correlated with BMI, WC, FAT%, TG, FPG, 2 h-PG, HbA1c, FIns, HOMA-IR, hs-CRP and TNF-α, IL-6 and MCP-1, but negatively with HOMA-β in all individuals ($P < 0.05$). Multiple linear regression analysis indicated that BMI, HOMA-IR, TNF-α and MCP-1 were independently associated with CCN3. Multivariate logistic regression analysis demonstrated that CCN3 was correlated with nT2DM. Finally, area under ROC curve of CCN3 (gender and age adjusted) for predicting the presence of nT2DM was 0.725(95% *CI*: 0.676–0.773). After an oral glucose challenge, there was no obvious change in the circulating levels of CCN3 as compared to 0 min ($P > 0.05$).

Conclusions: Elevation of CCN3 in nT2DM supports the hypothesis that CCN3 may serve as a risk factor associated with the pathogenesis of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is a significant attributable cause of mortality worldwide, which is mainly associated with insulin resistance (IR) and a systemic low-grade inflammatory state, especially in adipose tissue [1,2]. Adipose tissue is known to secrete a variety of active biological substances called adipokines, the aberrant adipokines production substantially contributes to IR and other prevalent metabolic disorders [3,4]. Although many adipokines have been found and characterized, the mechanisms by which adipokines regulate energy homeostasis and insulin sensitivity have not been fully understood.

The CCN3 gene was first identified in avian nephroblastomas as an integration site of the avian myeloblastosis-associated virus 1-N [5]. It encodes a peptide hormone that belongs to the CCN family of cell growth and differentiation regulators [6,7]. CCN3, also known as NOV (Nephroblastoma Overexpressed), is a circulating protein that could be detected in diverse human tissues and biological fluids, including the adrenal cortex, kidney, skeletal muscles, central nervous systems, heart, serum, amniotic fluid and cerebrospinal fluid, etc. [8]. Previous studies on CCN3 function were mainly focused on its role in organogenesis, inflammation, injury repair, fibrosis and apoptosis [9–12]. So far, there are very few reports concerning the serum concentration and function of CCN3 in diseases other than cancer.

Recently, researchers have highlighted CCN3 as a new member of adipose tissue derived cytokines, and shed light on key contributions of CCN3 in obesity-related metabolic disorders, low-grade inflammation and IR [13]. As a current clinical evidence indicated that the average plasma CCN3 concentration appeared to be significantly elevated in obese patients with a hyperlipidemia history, and exhibited a strong correlation with hs-CRP, BMI and fat mass [14]. More strikingly, a latest *in vivo* study also demonstrated that CCN3 deficiency could

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protect against obesity and improve insulin sensitivity, adipose tissue inflammation and metabolic profile in mice fed a high fat diet (HFD) [15]. In addition, genomics data have indicated that the CCN3 gene is localized on chromosome 8q24 [16], which is a susceptibility locus controlling β-cell function in linkage studies of patients with diabetes [17]. What's more, CCN3 is a novel transcriptional target of FOXO1 and it impairs insulin secretion in pancreatic β-cells [18]. In a word, the evidences listed above strongly supported that CCN3 may involved in IR and T2DM. However, to date, there has been no report demonstrating the relationship between CCN3 and IR in diabetic subjects.

To explore the clinical relevance of CCN3 in humans, we performed a cross-sectional study to explore serum CCN3 concentrations in normal subjects and newly diagnosed T2DM (nT2DM) patients, as well as analyzed its association with IR, anthropometric and metabolic parameters.

2. Methods

2.1. Participants

A total of 219 consecutive nT2DM patients were recruited from the First Affiliated Hospital of University of South China (Hengyang, China). T2DM was diagnosed according to oral glucose tolerance tests (OGTTs) and 1998 WHO diagnostic criteria. Subjects with nT2DM were not treated with hypoglycemic agents or insulin. Additionally, 205 age- and sex-matched healthy individuals who had undergone a routine physical examination were recruited as the control group. All participants completed a uniform questionnaire containing questions about the medical history and lifestyle factors (smoking and alcohol). Exclusion criteria included T1DM, patients with acute or chronic complications, hypertension, heart, hepatic or renal failure, or other known major diseases. The study was approved by the Human Research Ethics Committee (HREC) of the hospital, following the principles of the Declaration of Helsinki. Written informed consent was obtained from all subjects. Trial Registration: ChiCTR1800018347.

2.2. Anthropometric and biochemical measurements

Anthropometric parameters including height, weight, and waist circumference (WC) were measured using the standardized protocols. An analyzer of bioelectrical impedance was used to measure the percentage of body fat (FAT%). Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were determined by a specially assigned nurse using a mercury sphygmomanometer.

Subjects fasted overnight and blood samples were obtained at 8 to 9 am. A standardized clinical and laboratory procedure was used to evaluate the biochemical parameters. The lipid profile [Total cholesterol (TC), triglycerides (TG), low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol] was assessed using standard enzymatic methods. Fasting plasma glucose concentration (FPG) and 2h plasma glucose concentration (2h-PG) were measured by the hexokinase method. Glycosylated hemoglobin A1c (HbA1c) was measured by high-performance chromatography. High-sensitivity C-reactive protein (hs-CRP) was measured on the Hitachi 7600 analyzer (Kyoto, Japan). Fasting insulin concentration (FIns) was assayed using ELISA kits (DRG Company, Marburg, Germany). The homeostasis model assessment of insulin resistance (HOMA-IR) and homeostasis model assessment of insulin secretion (HOMA-β) were calculated using the following formula: HOMA-IR = FIns (μU/mL) × FPG (mmol/L)/22.5 and HOMA-β = FIns (mIU/l) × 20/[FPG (mmol/l) − 3.5].

2.3. Oral glucose tolerance test (OGTT)

After a 10–14-h overnight fast, a 75-g OGTT was also performed on 34 healthy subjects (16 man and 18 women). Blood samples were drawn at indicated time points for the measurements of blood glucose and serum CCN3.

Table 1
Clinical and biochemical features in study subjects.

Variables	Control	nT2DM	*P*-value
No. of subjects	205	219	–
Gender, M/F	96/109	108/111	0.609
Age (years)	43.50 ± 11.46	44.64 ± 8.53	0.245
Recent smoking (%)	15.6	23.7	0.036
Alcohol drinking (%)	11.7	12.8	0.735
BMI (kg/m^2)[a]	22.59 ± 2.07	25.04 ± 2.47	< 0.001
WC (cm)[a]	80 ± 6	90 ± 8	< 0.001
FAT (%)[a]	27.1 ± 3.1	30.5 ± 4.0	< 0.001
SBP (mmHg)[a]	121 ± 11	125 ± 9	< 0.001
DBP (mmHg)[a]	73 ± 8	79 ± 6	< 0.001
TC (mmol/l)[a]	4.49 ± 1.04	4.92 ± 1.26	< 0.001
TG (mmol/l)[a,b]	1.07 (0.77,1.52)	2.08 (1.38,3.08)	< 0.001
LDL cholesterol (mmol/l)[a]	2.36 ± 0.88	2.51 ± 1.06	0.128
HDL cholesterol (mmol/l)[a]	1.54 ± 0.38	1.28 ± 0.41	< 0.001
FPG (mmol/l)[a]	5.02 ± 0.39	11.34 ± 3.57	< 0.001
2h-PG (mmol/l)[a]	5.78 ± 0.43	18.73 ± 4.39	< 0.001
HbA1c (%)[a]	5.14 ± 0.39	9.65 ± 2.41	< 0.001
Fasting C-Peptide (ng/ml)[a]	1.66 ± 0.75	2.05 ± 0.75	< 0.001
FIns (mU/l)[a,b]	7.00 (5.82,8.20)	12.64 (10.58,15.36)	< 0.001
HOMA-IR[a,b]	1.58 (1.30,1.83)	5.85 (4.41,8.56)	< 0.001
HOMA-β[a,b]	74.47 (59.47,88.11)	37.50 (24.75,49.79)	< 0.001
hs-CRP(mg/l)[a,b]	1.56 (0.81,3.75)	2.34 (1.44,5.09)	< 0.001
TNF-α (ng/l)[a]	18.55 ± 5.11	25.78 ± 7.18	< 0.001
IL-6 (ng/l)[a]	11.67 ± 4.43	18.37 ± 6.15	< 0.001
MCP-1 (ng/l)[a]	108.69 ± 37.02	157.96 ± 47.40	< 0.001
CCN3(ng/ml)[a,b]	4.51 (3.55, 5.99)	6.71 (4.88, 8.56)	< 0.001

Values were given as means ± SD or median with interquartile range. nT2DM, newly diagnosed T2DM; BMI, Body mass index; WC, Waist circumference; FAT, Percentage of body fat; SBP, Systolic blood pressure; TC, Total cholesterol; TG, Triglycerides, LDL-C, Low-density lipoprotein cholesterol; HDL−C, High-density lipoprotein cholesterol; FPG, Fasting plasma glucose; 2h-PG, 2h plasma glucose; FIns, Fasting insulin; HOMA-IR, Homeostasis model assessment of insulin resistance; HOMA-β, Homeostasis model assessment of insulin secretion; hs-CRP, High-sensitivity C-reactive protein; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; MCP-1, Monocyte chemotactic protein-1.

[a] Adjusted for age and gender.

[b] Log transformed.

2.4. Measurements of CCN3 and inflammatory cytokines

Serum CCN3 levels were measured by the commercial ELISA kits from Boster Biological Technology (Pleasanton CA, USA, Catalog No. EK0833). Serum TNF-α IL-6 and MCP-1 levels were measured by ELISA kits from R&D systems (Minneapolis MN, USA & Canada, Catalog No.DTA00C, No.D6050, No.DCP00). The intra-assay and inter-assay variations were 4.8% and 5.3% for CCN3, 4.6% and 5.4% for TNF-α, 4.2% and 6.4% for IL-6, 7.8% and 6.7% for MCP-1, respectively. The measurement was performed following the instructions of the manufacturer. All samples were assayed in duplicate and random order.

2.5. Statistical analysis

Normal distributed data were expressed as mean ± SD. Data that were not normally distributed, as determined using Kolmogorox-Smirnov test, were logarithmically transformed before analysis and expressed as median with interquartile range (IQR). χ^2 and one-way ANOVA tests were used for comparison of categorical and continuous variables, respectively. Correlations between CCN3 and variables were assessed using *Pearson* correlation analyses by controlling for the covariates. Multiple linear regression was performed to determine variables that had independent associations with CCN3. The ORs for CCN3 levels and nT2DM were calculated by binary logistic regression. Receiver operator characteristic (ROC) curve analysis was employed to identify the optimal cut-off values of CCN3 to diagnose nT2DM. Row mean score test and Cochran-Armitage trend tests

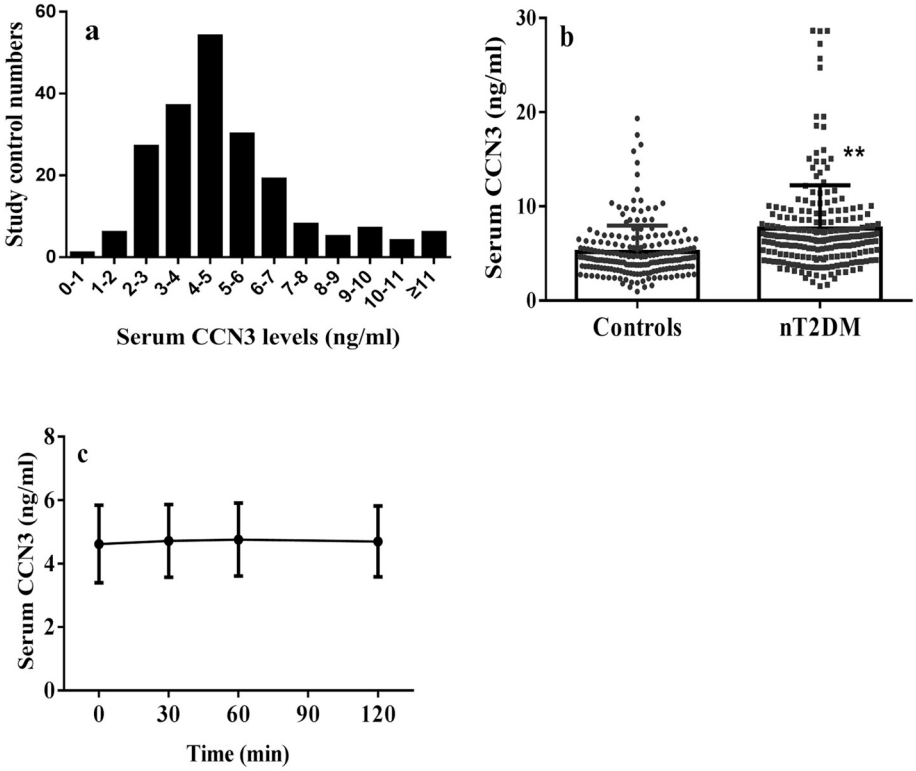


Fig. 1. Serum CCN3 levels in study subjects. a. Distribution of serum concentration of CCN3 in control subjects. b. Serum CCN3 levels in nT2DM patients and controls subjects. $^{**}P < 0.01$ *vs.* Control. c. Circulating CCN3 at different time points after oral glucose administration.

were conducted using SAS software version 9.30 (SAS Institute Inc., Cary, NC), while the other statistical analyses were conducted using SPSS software version 19.0 (SPSS, Chicago, IL). *P*-values < 0.05 (two-sided) were considered statistically significant.

3. Results

3.1. Characteristics and serum CCN3 levels of study participants

Anthropometric and metabolic parameters of the study subjects are showed in Table 1. The age and sex are comparable between controls and nT2DM patients. As expected, Recent smoking(%), SBP, DBP, BMI, WHR, FAT%, TC, TG, FPG, 2 h-PG, HbA1c(%), Fasting C-Peptide, FIns, HOMA-IR, hs-CRP, TNF-α, IL-6 and MCP-1 were significantly increased, whereas HDL-C and HOMA-β were significantly decreased in nT2DM subjects when compared with the controls ($P < 0.01$). However, there were no significant differences in Alcohol drinking (%) and LDL-C between control and nT2DM groups.

In addition, serum CCN3 levels were also assayed in all subjects. The distribution of serum CCN3 was displayed in Fig. 1a. Serum CCN3 concentrations were located from 0.97 to 7.0 ng/ml for most control subjects (85.4%). Importantly, nT2DM patients had higher serum CCN3 levels than the control subjects [6.71(4.88, 8.56) *vs.* 4.51(3.55, 5.99) ng/ml, $P < 0.01$; Fig. 1b]. There was no significant gender difference (5.09[3.89,6.93] *vs.* 5.84[4.09,7.88]) ng/ml for males and females respectively, $P = 0.060$). By further subgroup analysis, we found that there was no significant difference in serum CCN3 levels between males and females in the control group (4.50 [3.58, 5.84] *vs* 4.60 [3.44, 6.15] ng/ml, $P = 0.606$), however, compared with males, serum CCN3 levels in female were significantly elevated in the nT2DM group (6.54 [4.40, 7.88] *vs* 6.97 [5.75, 9.18] ng/ml, $P = 0.012$).

3.2. Correlation of CCN3 with clinical parameters in study subjects

Next, we investigated the association of serum CCN3 levels with various other parameters. Serum CCN3 correlated positively with adiposity-related parameters (BMI, WC, FAT%, $P < 0.05$) and glucose metabolic parameters (FPG, 2 h-PG, HbA1c, FIns, $P < 0.01$) (Table 2). It also correlated with TG and insulin resistance indices (FIns and increased HOMA-IR), $P < 0.01$; Table 2). In addition, serum CCN3 levels correlated negatively with HOMA-β ($P < 0.01$; Table 2). Importantly, serum CCN3 also correlated positively with inflammatory indices (hs-CRP, TNF-α, IL-6 and MCP-1, $P < 0.01$; Table 2). All these correlations remained similar after further adjustment for age and gender. The results of multiple stepwise regression showed that only BMI, HOMA-IR, TNF-α and MCP-1 were independently related factors to serum CCN3 levels ($Y_{Ln[CCN3]} = 0.690 + 0.037 \times BMI + 0.043 \times HOMA\text{-}IR + 0.008 \times TNF\text{-}\alpha + 0.001 \times MCP\text{-}1$, $R^2 = 0.244$, $P < 0.001$; Table 2).

3.3. The effect of serum CCN3 on the incidence of nT2DM

Binary logistic regression analysis showed that serum CCN3 concentrations were significantly associated with nT2DM even after controlling for covariates (Table 3). The unadjusted OR for the incidence of nT2DM was 2.434(95% *Cl*: 1.499–3.952, $P < 0.001$) for the middle tertile and 6.523(95% *Cl*: 3.870–10.955, $P < 0.001$) for the highest tertile when compared with the lowest one. With the lowest tertile as the reference, after controlling for age, gender, recent smoking, BMI, TC, TG, LDL-C, HDL-C, hs-CRP, TNF-α, IL-6 and MCP-1 (Model 4), the adjusted OR of the middle tertile was 1.896 (95% *Cl*: 0.893–4.025, $P = 0.096$) and 3.730(95% *Cl*, 1.705–8.162, $P = 0.001$) for the highest tertile. Furthermore, when concentrations were analyzed both by a Row Mean Scores test and a Cochran-Armitage trend test, the increasing CCN3 levels showed a significant linear trend and were independently associated with nT2DM (Table 4). The ROC curves analyses revealed that the best cutoff value for serum CCN3 (gender and sex adjusted) to predict nT2DM was 5.77 ng/ml (sensitivity 70.3%, specificity 73.2%, AUC 0.725[0.676, 0.773], $P < 0.001$; Fig. 2).

Table 2
Correlation of serum Ln(CCN3) levels with clinical variables in all subjects.

Variable	Simple		Multiple	
	r	*P*-value	*β* ± SE	*P*-value
Age(years)	0.060	NS	0.002 ± 0.002	0.421
Recent smoking (%)	0.005	NS		
Alcohol drinking (%)	−0.041	NS		
BMI (kg/m^2)	0.342	< 0.001	0.037 ± 0.010	< 0.001
WC(cm)[b]	0.272	< 0.001		
FAT%	0.242	< 0.001	0.005 ± 0.007	0.409
SBP (mmHg)	0.095	NS		
DBP (mmHg)	0.094	NS		
TC (mmol/l)	0.051	NS		
TG (mmol/l)[a]	0.158	0.001	−0.028 ± 0.016	0.088
HDL-C(mmol/l)	−0.089	NS		
LDL-C(mmol/l)	0.049	NS		
FPG (mM)	0.291	< 0.001	−0.022 ± 0.015	0.141
2 h-PG(mM)	0.324	< 0.001	−0.006 ± 0.009	0.538
HbA1c (%)	0.293	< 0.001		
Fasting C-Peptide (ng/ml)	0.095	NS		
FIns (mU/l)[a]	0.253	< 0.001	−0.008 ± 0.011	0.445
HOMA-IR[a]	0.337	< 0.001	0.043 ± 0.018	0.015
HOMA-β[a]	−0.289	< 0.001	−0.005 ± 0.002	0.099
hs-CRP(mg/l)[a]	0.197	< 0.001	0.006 ± 0.003	0.052
TNF-α(ng/l)	0.326	< 0.001	0.008 ± 0.004	0.034
IL-6(ng/l)	0.278	< 0.001	0.002 ± 0.005	0.670
MCP-1(ng/l)	0.288	< 0.001	0.001 ± 0.001	0.023

In multiple linear regression analysis, values included for analysis were age, BMI, Fat%, TG, FPG, 2h-PG, FIns, HOMA-IR, HOMA-β, hs-CRP, TNF-α, IL-6 and MCP-1.
BMI, Body mass index; WC, Waist circumference; FAT, Percentage of body fat; SBP, Systolic blood pressure; TC, Total cholesterol; TG, Triglycerides, LDL-C, Low-density lipoprotein cholesterol; HDL−C, High-density lipoprotein cholesterol; FPG, Fasting plasma glucose; 2h-PG, 2h plasma glucose; FIns, Fasting insulin; HOMA-IR, Homeostasis model assessment of insulin resistance; HOMA-β, Homeostasis model assessment of insulin secretion; hs-CRP, High-sensitivity C-reactive protein; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; MCP-1, Monocyte chemotactic protein-1.
[a] log transformed before analysis.
[b] WC did not enter into the multivarate regression due to its high inter-correlation with FAT%.

Table 3
Logistic regression analysis of the association of serum CCN3 with nT2DM.

	CCN3		
	T1 (*n* = 142)	T2 (n = 142)	T3 (*n* = 140)
CCN3 (μg/L)	< 4.44	4.44–6.82	> 6.82
nT2DM, n (%)	42 (30.0)	73 (51.4)	104 (74.3)
Model 1	Reference	2.434(1.499,3.952) *P* < 0.001	6.523(3.870, 10.955) *P* < 0.001
Model 2	Reference	2.412(1.481, 3.929) *P* < 0.001	6.663(3.923, 11.317) *P* < 0.001
Model 3	Reference	2.283(1.224, 4.264) *P* < 0.001	3.715(1.860, 7.420) *P* < 0.001
Model 4	Reference	1.896(0.893, 4.025) *P* = 0.096	3.730(1.705, 8.162) *P* = 0.001

Model 1: crude model.
Model 2: Model 1 + Age, Gender, recent smoking.
Model 3: Model 2 + BMI, TC, TG, LDL-C, HDL-C.
Model 4: Model 3 + TNF-α, hs-CRP, IL-6, MCP-1.
BMI, Body mass index; TC, Total cholesterol; TG, Triglycerides, LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; hs-CRP, High-sensitivity C-reactive protein; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; MCP-1, Monocyte chemotactic protein-1.

3.4. Circulating CCN3 levels in response to glucose challenge in healthy individuals

To determine whether blood glucose *per se* could modulate serum CCN3 levels *in vivo*, we examined the effects of glucose challenge on CCN3 in 34 healthy subjects. During the 2-h OGTT, in response to oral glucose challenge-induced hyperglycemia and hyperinsulinemia, there was no obvious change in the circulating levels of CCN3 as compared to 0 min (0 min: 4.65 ± 1.60 ng/ml; 30 min: 4.73 ± 1.56 ng/ml; 60 min: 4.73 ± 1.47 ng/ml; 120 min: 4.69 ± 1.46 ng/ml, $P > 0.05$, Fig. 1c).

Table 4
Row mean scores and Cochran-Armitage trend test of the impact of CCN3 on nT2DM.

	nT2DM	
	χ^2	*P*-value
ROW Mean Scores Test	31.835	< 0.001
Cochran-Armitage Test	44.593	< 0.001

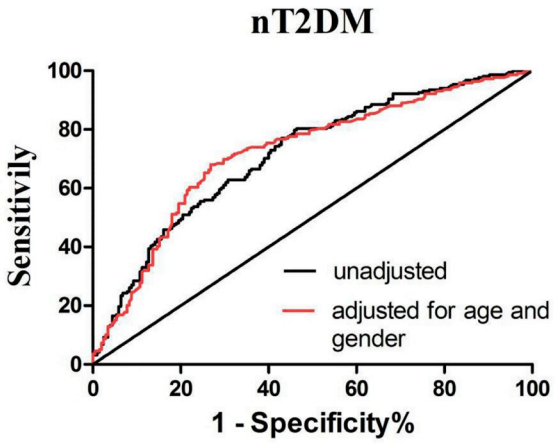
4. Discussion

In recent years, some *in vivo* and *in vitro* studies have documented that CCN3 is involved in the modulation of energy metabolism, systemic low-grade inflammation and IR, while the clinical relevance of these findings has rarely been investigated. To the best of our knowledge, this is the first study to examine the serum concentration of CCN3 in newly diagnosed T2DM (nT2DM) in humans.

The main findings of this study are as follows: i) serum levels of CCN3 in nT2DM are significantly higher compared with healthy controls. ii) serum levels of CCN3 were positively correlated with BMI, WC, FAT%, TG, FPG, 2 h-PG, FIns, HbA1c, HOMA-IR, hs-CRP, TNF-α, IL-6 and MCP-1 but inversely correlated with HOMA-β in all individuals. iii) BMI, HOMA-IR, TNF-α, and MCP-1 were independently associated with serum CCN3 in nT2DM. iv) Serum levels of CCN3 equal to or > 5.77 ng/ml were observed to be the optimal cutoff for differentiating T2DM patients from healthy controls.

In 2003, Thibout et al. developed the first enzyme immunoassay specific to human CCN3 allowing the detection of CCN3 protein in biological fluids, such as serum, amniotic fluid and cerebrospinal fluid [19]. However, to date, only one study have reported an elevated plasma CCN3 concentration in patients with metabolic disorders [14]. Consistently, animal studies have provided evidences that the adipose tissues gene expressions of CCN3 were increased in obesity-induced and genetic diabetic mice [14,20]. What's more, CCN3$^{-/-}$ knockout mice in the presence of a high fat diet, were less prone to gain weight and less insulin resistant (IR) than wild type mice, suggesting CCN3 may serve as a risk factor associated with the pathogenesis of T2DM [15]. Nevertheless, Riser and colleagues showed that the renal cortex mRNA level of CCN3 was little or no measurable early but dramatically increased in the late stage diabetic mice and they indicated CCN3 was protective in diabetic renal disease models [21]. CCN3 was shown to negatively regulate the profibrotic family member CCN2 expression to inhibit key fibrosis markers expression (CCN2, Col 1a2,TGF-β1, and PAI-1) in a mesangial cell model of Diabetic nephropathy (DN) [21]. Thus, CCN3/CCN2 ration might be as a marker of reflecting fibrosis degree of DN.

These contradictory findings would question the expression and role of CCN3 in the different stage of T2DM. Here, we established for the first time that serum CCN3 levels were markedly elevated in nT2DM. Combined with the findings from CCN3 knockout studies, CCN3 might be responsible for the initiation of T2DM through induction IR and impairment pancreatic beta cell function [15,18]. In compared to early diabetes, DN is a major diabetic complication which is characterized with pronouced extraglomerular fibrosis mediated with TGF-β signaling pathway. Stimulation of TGF-β response by long-term hyperglycemia could inhibit CCN3 expression while enhance CCN2 expression, thereby exacerbating fibrosis process in DN [21]. However, supplement of CCN3 protein could in turn block TGF-β siganling pathway to reverse fibrosis development in DN mouse [22]. This discrepancy of CCN3 expression is likely due to the serum and adipose tissues CCN3 mRNA



Variables	Area	*P*-value	95% Confidence Interval	
			Lower limit	Upper limit
CCN3	0.721	< 0.001	0.672	0.770
CCN3(age and gender adjusted)	0.725	< 0.001	0.676	0.773

Fig. 2. ROC curve analysis was performed for the prediction of nT2DM according to the CCN3 levels.

levels independently of its renal cortex mRNA level. Besides, we still can't rule out the possibility that the increased CCN3 levels in circulation in obese and IR humans and mice might be served as a defensive response to adapt to adiposity, IR, inflammation or other metabolic disorders. Further studies are still needed to elucidate this point.

Obesity is linked to hyperinsulinemia and insulin resistance, and is an independent risk factor for the onset of T2DM [23,24]. Given that CCN3 may play a critical role in the development of obesity [13], we first examined whether the adiposity-related parameters were related to plasma CCN3. The data indicated that serum CCN3 has a strong relationship with adiposity-related parameters in all individuals, including BMI, WC and FAT%. Of significance, multiple stepwise regression analysis demonstrated that BMI was independently associated with CCN3 concentration. The results were in agreement with other clinical study, which provided for the first time evidence that BMI were major independent determinants of plasma CCN3 [14]. Moreover, an *in vivo* study also demonstrated that a striking decrease of body weight ratios was observed in CCN3$^{-/-}$ mice when fed with a HFD not a regular chow compared with the controls [15], further pinpointed CCN3 is not sufficient to induce an adiposity, while it may be only a factor that promotes an excess in adiposity when exposed to an obesogenic condition.

Adipose tissue has the endocrine role to regulate metabolism and balance energy homeostasis [25]. Several adipose tissue-secreted cytokines can either enhance or impair insulin action [26–29]. In the current study, our data clearly showed that CCN3, a newly described adipokine, was strongly and positively correlated with the well-known indices of T2DM in all individuals. Among these indices, HOMA-IR was independent related factors with serum CCN3 levels. However, in our ROC curve analysis, the results show that circulating CCN3 may predict nT2DM in our study population. In one aspect, the range of AUC (0.725) was considered to be mild-to-moderate significance, which may be due to the influence of the sample size and a non-normal distribution of CCN3 levels in the studied population. Hence, the circulating CCN3 may not be a good marker for predicting nT2DM. Although the exact mechanisms linking increased CCN3 levels to IR and T2DM cannot be ascertained based on our study, it may be related to the role of CCN3 in regulating glucose metabolism and insulin signaling pathways that have been illuminated by both rodent and cell studies.

Studies during the past decades have established that chronic low-grade inflammation is a crucial feature of T2DM [30]. TNF-α, IL-6 and MCP-1 are important marker of chronic low-grade inflammation and is also known to play a key role in the pathogenesis of IR and T2DM [31–33]. Dramatically, several lines of evidences have highlighted that CCN3 is closely linked to inflammation, which expression can be directly regulated by TNF-α [8,12]. In addition, it has been shown that CCN3 can act directly on cultured adipocytes to promote CCL2/MCP-1 synthesis and secretion [15]. Moreover, an animal-based study also points out that CCN3-induced chemokines secretion could enable immune cell recruitment and inhibit M2 phenotype macrophages [15]. In line with the previous data, we found that CCN3 concentrations were positively associated with hs-CRP, TNF-α IL-6 and MCP-1 in all individuals. In addition, TNF-α and MCP-1were independent factor related to serum CCN3 levels. Based on these findings, one can speculate that circulating CCN3 may also be a marker of chronic inflammation.

Even though lipid metabolism disorder are often associated with impaired glucose tolerance and T2DM [34]. However, we observed that only TG was positively but weakly associated with the CCN3 level (Table 2). Additionally, we did not find any relation of CCN3 with TC, HDL-C and LDL-C. These data suggest that CCN3 may not be an ideal biomarker for predicting dyslipidemia.

Next, to investigate whether blood glucose *per se* could modulate serum CCN3 levels, we observed the dynamic changes of CCN3 in response to a glucose challenge in healthy individuals. Unexpectdely, serum CCN3 concentrations didn't exhibited a similar change with blood glucose, suggesting blood glucose could not modulate serum CCN3 levels directly.

There were several limitations in our current study. First, the cross-sectional design of this study does not allow us to deduce a causal relationship between CCN3 and nT2DM, we still can not rule out the elevation of circulating CCN3 in nT2DM individuals might be a compensatory up-regulation. Second, whether CCN3 could serve as a diagnostic marker between T2DM with other types of diabetes (T1DM, LADA, pancreatic diseases, endocrinopathies, et al.), or in people at risk for T2DM but still non diabetic remains to be investigated. Moreover, CCN3 may have an interest for the subsequent onset of insulin-resistance-related cardiometabolic complications (prognosis value of CCN3 levels). Further large-scale prospective studies have been taken into our consideration.

5. Conclusions

In summary, our data showed that serum CCN3 levels were increased in patients with nT2DM. BMI, HOMA-IR and TNF-α were

independent related factors with serum CCN3 levels. We speculate that adipokine CCN3 may serve as a risk factor associated with the pathogenesis of T2DM. However, more functional studies are required to determine the molecular details of metabolism regulation by CCN3.

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Conflicts of interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2019.03.006.

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