Clinical Evaluation of Serum Complement Component 1q and Cystatin C in the Diagnosis of Early Diabetic Kidney Disease

Type
Research paper

Keywords
cystatin C, creatinine, neutrophil gelatinase-associated lipocalin, early diabetic kidney disease, complement component 1q

Abstract

Introduction
To investigate the diagnostic value of serum complement components 1q (C1q), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C (CysC), creatinine (SCr) and urea (SUr) for early diabetic kidney disease (EDKD)

Material and methods
Individuals with type 2 diabetes mellitus (DM) and healthy subjects at Mianyang Central Hospital from March, 2017 to November, 2017 were enrolled according to the inclusion and exclusion criteria. Serum SUr, SCr, CysC, C1q and NGAL were detected. Diagnostic values were evaluated by ROC.

Results
Totally, 546 individuals were enrolled, including 136 patients in simple DM group (SDM group), and 109 patients in EDKD group and 301 healthy controls (HC group). Compared with SDM group and HC group, serum SUr, SCr, C1q, CysC and NGAL in EDKD group were significantly increased (P<0.01). C1q, CysC, and NGAL were significantly related to EDKD and they were risk factors for EDKD. Diagnostic performance of CysC (AUC=0.813, 0.777~0.845), C1q (AUC=0.797, 0.761~0.830) or NGAL (AUC=0.770, 0.732~0.805) was much higher than that of SCr (AUC=0.681, 0.640~0.720) or SUr (AUC=0.650, 0.608~0.690) (p < 0.05). C1q +CysC (AUC=0.896, 0.867~0.920; Se=77.98%, 74.27% - 81.39%; Sp=89.4%, 86.59% - 91.92%; YI=0.675) had the optimal diagnostic performance. However, combination of C1q+CysC+NGAL (AUC=0.909, 0.882~0.932; Se=88.99%, 86.06% - 91.49%; Sp=80.32%, 76.73% - 83.57%; YI=0.693) or C1q+CysC+NGAL+SCr (AUC=0.912, 0.885~0.934; Se=87.16%, 84.06% - 89.85%; Sp=83.98%, 80.63% - 86.96%; YI=0.711) did not further improve diagnosis performance for EDKD (p>0.05).

Conclusions
C1q, CysC and NGAL have similar diagnostic performances, which is superior to SCr, in diagnosis of EDKD. Moreover, C1q +CysC combination has the optimal diagnostic performance for EDKD.
Clinical Evaluation of Serum Complement Component 1q and Cystatin C in the Diagnosis of Early Diabetic Kidney Disease

Jiafu Feng1**, Lingying Fan2#, Yuwei Yang1

1Department of Clinical Laboratory, Mianyang Central Hospital, Affiliated to Southwest Medical University, Mianyang 621000, Sichuan, China;
2Department of Clinical Laboratory, Southwest Medical University, Luzhou 646000, Sichuan, China.

#These authors contributed equally to this work.

*Corresponding author:
Jiafu Feng
Department of Clinical Laboratory, Mianyang Central Hospital, Affiliated to Southwest Medical University
No.12 Changjiaxiang, Jingzhong Street, Mianyang 621000, Sichuan, China.
Tel: +86-0816 2228 572
Email: jiafufeng@aliyun.com

Running title: The diagnosis of early diabetic kidney disease

Abstract

Introduction: The aim of the study was to investigate the diagnostic value of serum complement components 1q (C1q), neutrophil gelatinase-associated lipocalin (NGAL), cystatin C (CysC), creatinine (SCr) and urea (SUr) for early diabetic kidney disease (EDKD).
Material and methods: Individuals with type 2 diabetes mellitus (DM) and healthy subjects at Mianyang Central Hospital from March, 2017 to November, 2017 were enrolled. Totally, 546 individuals were enrolled, including 136 patients in simple DM group (SDM group), 109 patients in EDKD group and 301 healthy controls (HC group). Patients with type 2 DM diagnosed according to the American Diabetes Association (ADA) guidelines and with eGFR≥60 mL/min/1.73m² were included. Non-DM patients who had kidney impairment were excluded. Serum SUr, SCr, CysC, C1q and NGAL were detected. Diagnostic values were evaluated by receiver operating characteristic curve.

Results: Compared with SDM group and HC group, serum SUr, SCr, C1q, CysC and NGAL in EDKD group were significantly increased (P<0.01). C1q, CysC, and NGAL were related to EDKD and they were risk factors for EDKD. Diagnostic performance of CysC (AUC=0.813, 0.777–0.845), C1q (AUC=0.797, 0.761–0.830) or NGAL (AUC=0.770, 0.732–0.805) was much higher than that of SCr (AUC=0.681, 0.640–0.720) or SUr (AUC=0.650, 0.608–0.690) (p < 0.05). C1q+CysC [AUC=0.896, 0.867–0.920; Se=77.98%, 74.27% - 81.39%; Sp=89.4%, 86.59% - 91.92%; Youden index (YI) =0.675] had the optimal diagnostic performance. However, combination of C1q+CysC+NGAL (AUC=0.909, 0.882–0.932; Se=88.99%, 86.06% - 91.49%; Sp=80.32%, 76.73% - 83.57%; YI=0.693) or C1q+CysC+NGAL+SCr (AUC=0.912, 0.885–0.934; Se=87.16%, 84.06% - 89.85%; Sp=83.98%, 80.63% - 86.96%; YI=0.711) did not further improve diagnosis performance for EDKD (p>0.05).
Conclusion: C1q, CysC and NGAL have similar diagnostic performances, which is superior to SCr, in diagnosis of EDKD. C1q +CysC combination has the optimal diagnostic performance for EDKD.

Key words: early diabetic kidney disease; complement component 1q; cystatin C; neutrophil gelatinase-associated lipocalin; creatinine
1. Introduction

Diabetes mellitus (DM) is a chronic life-threatening disease and its incidence is increasing in recent decades [1]. According to the latest report of the International Diabetes Federation, about 425 million adults worldwide suffered from DM in 2017, and the death caused by DM accounted for 10.7% of all-cause deaths [2]. It is estimated that by 2045, DM patients worldwide will reach 629 million [2]. Recent study has shown that the incidence of diabetes in China is 10.9% [3]. Diabetic kidney disease (DKD), also termed chronic kidney disease (CKD), is caused by DM (both type 1 and type 2), and early DKD (EDKD) refers to the early damage of kidney in diabetic patients [4]. Approximately 10%-30% of diabetic patients develop DKD because of microangiopathy and DKD is one of the main causes of end-stage kidney disease in developed countries [5]. DM patients complicated with DKD have reduced life quality and increased mortality risk [6]. The diagnosis of DKD is mainly based on the Clinical Practice Guideline of Chronic Kidney Disease in KDIGO 2012 [7]. Persistent albuminuria (urinary albumin excretion rate or urine albumin-to-creatinine ratio as an indicator of evaluation) for more than three months is globally recognized as the main diagnostic criteria for DKD (including EDKD) when there is no histological diagnosis [8, 9]. Thus, the diagnosis of DKD/EDKD is difficult. Therefore, early diagnosis, early prevention and early intervention are critical to delay the occurrence and/or development of DKD (especially in the early stage of kidney damage), improving the survival rate and life quality of DM patients. In laboratory medicine, searching for
biomarkers related to DKD, especially EDKD, may provide new directions for DKD diagnosis and treatment [10].

Serum creatinine (SCr) and serum Urea (SUR), especially SCr, are clinically used indicators for evaluating renal function, which has been recommended by 2012 Kidney Disease Outcome Quality Initiative (KIDGO). However, it is well-known that SCr may not change significantly in the early stage of kidney impairment, so its diagnostic sensitivity for chronic kidney disease (including EDKD) is low [11, 12]. Serum creatinine increased significantly when kidney function decreased by 50% [13]. Some studies demonstrated that urinary albumin to creatinine ratio (UACR) can better reflect the early damage of the kidney and can replace the quantitative test of 24-h urinary albumin [7,14,]. Nevertheless, the accurate detection of UACR is difficult due to the lack of urine-specific calibration [15, 16].

Cystatin C (CysC) is a recently identified biomarker that could reflect glomerular filtration function with higher sensitivity and specificity than SCr [17]. The CysC level can be used to directly calculate the estimated glomerular filtration rate (eGFR) of CKD patients independent of other factors [18-20]. Complement component 1q (C1q) is an important antigen recognition molecule in the classical activation pathway of the complement system [21]. It plays an important role in maintaining self-tolerance and regulating inflammatory responses [21]. Studies have shown that C1q is associated with the development of type 2 diabetes mellitus (T2DM) and its expression is markedly altered in some kidney diseases [22-24]. Neutrophil gelatinase-associated lipocalin
(NGAL) is a small molecule that regulates the apoptosis of renal tubular epithelial cells [25-27]. NGAL is barely detected in renal tissues under normal conditions, but its expression is significantly increased during renal tubulointerstitial neutrophil proliferation, with good sensitivity and specificity for acute kidney injury (AKI) [26-28]. Moreover, the Workgroup Statements from the Tenth Acute Dialysis Quality Initiative (ADQI) Consensus Conference considers NGAL as the most potent marker of acute kidney injury [28]. Nevertheless, the reports on the application of C1q, CysC and NGAL in the diagnosis of DKD are rare.

Therefore, in this study, we investigated the clinical value of the above mentioned traditional and novel renal function biomarkers (including CysC, NGAL, C1q, SUr and SCr) for the diagnosis of EDKD.
2. Material and Methods

2.1. Patients and healthy controls

This is a case-control study and a prospective study. This study was approved by the Human Ethics Committees Review Board at the Mianyang Central Hospital (S2014048, Nov. 25, 2014), and written informed consent was obtained from all of the participants prior to their enrollment.

A total of 546 subjects were included in this study, of whom 245 were T2DM patients and 301 were healthy controls.

(1) Patients: A total of 245 individuals with T2DM at Mianyang Central Hospital from March, 2017 to November, 2017 were enrolled consecutively into this study. Because of the large number of patients, the medication methods of T2DM patients were very complex. There were single drug application and combination of two drugs. The drugs and administration methods were as follows: Metformin Hydrochloride Sustained Release Tablets (0.5-1.0g, Q.d., Qingdao Huanghai Pharmaceutical Co., Ltd.), Repaglinide (2mg, Qid., Novo Nordisk A/S, Production of Boehringer Ingelheim Pharma GmbH & Co.KG, Germany), Gliclazide Tablets (80mg, Q.d., Guangzhou Baiyunshan Guanhua Pharmaceutical Co., Ltd. ), Glurenor (30 mg, Q.d./60mg, Bid./90mg, Tid. Beijing Wanhui Shuanghe Pharmaceutical Co., Ltd.), and/or Isophane protamine biosynthetic human insulin injection [300IU, Q.d., Denmark, Novo Nordisk A/S sub-package: Novo Nordisk (China) Pharmaceutical Co., Ltd.].

Inclusion criteria: patients diagnosed with T2DM according to the American
Diabetes Association (ADA) “Standards of Medical Care in Diabetes” [16], as well as
with HbA1c≥6.5% or fasting glucose≥7.0mmol/L or 2h glucose≥11.1mmol/L during an
OGTT or random glucose ≥11.1 mmol/L in a patient with classic symptoms of
hyperglycemia or hyperglycemic crisis. In addition, the patients' eGFR was ≥60
mL/min/1.73m².

Exclusion criteria: patients with infectious diseases, cerebral infarction,
myocardial infarction, hypertension, anemia, benign and malignant tumors, connective
tissue disease, allergy, systemic lupus erythematosus, rheumatoid arthritis, thyroid
disease or morbid obesity, or other systemic or active diseases, or treated with
potentially nephrotoxic drugs, were excluded; patients with known non-DKD (i.e.,
interstitial kidney disease, polycystic kidney disease, membranous kidney disease,
kidney stones, autosomal dominant tubulointerstitial kidney disease, minimal change
disease, IgA nephropathy, IgG4-related kidney disease, and tubulointerstitial nephritis)
were excluded; Menstrual women as well as pregnant women were also excluded.

According to the Clinical Practice Guideline of Chronic Kidney Disease in
KDIGO 2012 [7], patients were divided into single T2DM group (SDM; T2DM patients
with UACR < 30 mg/g and eGFR ≥ 90 mL/min/1.73m²) and EDKD group (T2DM
patients with 30 ≤ UACR <300 mg/g and eGFR ≥60 mL/min/1.73m² or UACR <30
mg/g and 60 ≤ eGFR ≤ 90 mL/min/1.73m²) according to the results of UACR and
eGFR.

(2) Healthy controls: the volunteers who came to the hospital for physical
examination were tested for eGFR and were divided into two groups accordingly (<90 ml·min⁻¹·1.73 m⁻² or ≥90 ml·min⁻¹·1.73 m⁻²). Because the eGFR of most healthy individuals are ≥90 ml·min⁻¹·1.73 m⁻², a stratified random sampling is carried out according to the proportion of about 1:3. Finally, 301 healthy subjects (eGFR range 72.37–147.71 ml·min⁻¹·1.73 m⁻²) were selected as the control. Their liver and kidney function were normal. The results of routine blood and urine tests were within normal range.

Liver and kidney function were evaluated by the following indicators (method, reference ranges): alanine transaminase (continuous monitoring method, 9-50U/L), aspartate transaminase (continuous monitoring method, 15-40U/L), gamma-glutamyl transpeptidase (continuous monitoring method, 10-60U/L), total bilirubin (diazoo method, 0-26μmol/L), direct bilirubin (diazoo method, 0-6.8μmol/L), total protein (biuret method, 65-85g/L), albumin (bromocresol green methods, 40-55g/L), urea (urease/glutamate-dehydrogenase method, 2.86-8.20mmol/L), creatinine (sarcosine oxidase method, 57-97μmol/L for male, 41-73μmol/L for female), Cystatin C (particle enhanced immunoturbidimetic method, 0.47-1.09mg/L). The above indexes were tested by a LABOSPECT008 automatic biochemical analyzer (Hitachi, Japan) with corresponding kits (Maccura Biotechnology Co., Ltd., Sichuan, China). Subjects with the levels of these indicators in normal range were considered to have normal liver and kidney functions.

For blood routine: All whole blood samples were assayed on the Sysmex XN-10...
haematology analyzer (Kobe, Japan) for the following indicators (reference ranges):

- Haemoglobin: 130–165 g/L and 115–155 g/L; red blood cell count: 4.30–5.80 \times 10^{12}/L and 3.80–5.10 \times 10^{12}/L; haematocrit: 0.40–0.50 L/L and 0.37–0.47 L/L, for males and females, respectively; mean corpuscular volume: 82–100 fL; mean corpuscular haemoglobin: 27–34 pg; mean corpuscular haemoglobin concentration: 316–354 g/L; red blood cell distribution width: 12.2–14.8%; platelet count: 100–300 \times 10^{9}/L; white blood cell count: 5.0–9.5 \times 10^{9}/L; neutrophil count: 1.80–6.30 \times 10^{9}/L; lymphocyte count: 1.10–3.20 \times 10^{9}/L; monocyte count: 0.10–0.60 \times 10^{9}/L; eosinophil count: 0.02–0.52 \times 10^{9}/L; and basophil count: 0.00–0.06 \times 10^{9}/L. Subjects with the levels of these indicators in normal range were considered to have normal blood routine results.

For urine routine: The urine visible components were determined by a Sysmex UF-5000 flow cytometer (Kobe, Japan), and the urine protein and pH were determined by reagent strip urinalysis method. The reference ranges were as follows: white blood cell count, 0-25cell/L; red blood cell count, 0-20 cell/L; epithelial cell count, 0-20cell/L; casts, none; pH, 5.0-8.0; protein, negative. Subjects with the levels of these indicators in normal range were considered to have normal urine routine results.

2.2. Sample collection

A total of 5 mL venous blood samples were collected from each individual after fasting overnight for 8-14 hours. Samples were then centrifuged at 3600 g at 4°C for 15 min and the serum was isolated for the detection of SUr, SCr, C1q and NGAL. All
samples of hemolysis, lipemia or jaundice were considered unqualified and were excluded.

After blood sample collection, about 10 mL of the middle part of the urine was collected from each subject with a disposable urine collection tube (Huajie HJ1011, Jiangsu, China). Urine samples were centrifuged at 1500 g for 10 min. The supernatant was collected and used to determine the urine creatinine (UCr) and urinary albumin (UA) content within 2 h.

2.3. Sample measurement

CysC and NGAL were measured by particle enhanced immunoturbidimetic method. C1q was measured by immunoturbidimetic method. SUr was measured by urease/glutamate-dehydrogenase method and SCr was measured by sarcosine oxidase method. Fasting glucose was detected by Hexokinase method. The LABOSPECT008 automatic biochemical analyzer (Hitachi, Japan) was then used. The C1q kit was purchased from Shanghai Beijia Biochemical Reagent Co., Ltd. (China). The kits for glucose, CysC, NGAL, SUr and SCr analysis were purchased from Maccura Biotechnology Co., Ltd. (Sichuan, China). Urine creatinine was measured by sarcosine oxidase method and urinary albumin was measured by modified immunoturbidimetric assay using an A25 analyzer (BioSystems, Spain). The kits for urine creatinine and urinary albumin detection were purchased from Biostec Biotechnology Co., Ltd. (Chongqing, China).

The eGFR was expressed in mL/min/1.73 m$^2$ and calculated according to the
formula for Chinese population: eGFR = 78.64\times \text{CysC}^{-0.964} \ [29]. UACR is the ratio of UAlb to UCr, which is calculated as UACR (mg/gCr) = \frac{\text{UAlb} (\text{mg/L})}{\text{UCr} (\text{g/L})}.

In this study, the reference values were validated by C28-A3 of Clinical and Laboratory Standards Institute and ISO15189 rules, which were as follows: CysC: 0.51-1.09mg/L; NGAL: 0-100.0μg/L; C1q: 159.0-233.0mg/L; SUr: 2.86-8.20mmol/L; SCr: 57.0-97.0μmol/L (male), 41.0-73.0μmol/L (female); UACR: <30.0mg/gCr.

2.4. Diagnostic performance evaluation

The diagnostic performance of each single index of CysC, C1q, NGAL, and SCr were evaluated by receiver operating characteristic curve (ROC) analysis. In clinic, most doctors often choose a combination of multiple indicators to evaluate kidney function. Therefore, we also evaluated the combined diagnostic performance of C1q, NGAL, CysC and SCr with ROC. The combination among the indexes is indicated as “and (+)”. The aim is to find the optimal combination of these traditional and new renal function indicators for the diagnosis of EDKD.

2.5. Statistical analysis

All data were analyzed using IBM SPSS Statistics, version 20.0 (IBM Corporation Armonk, NY, USA), or MedCalc15.2.2 (Medcalc Software, Ostend, Belgium). P-values less than 0.05 were considered statistically significant. When multiple comparisons of three groups were performed, the P-value was adjusted by Bonferoni, and P-values less than 0.0167 were considered statistically significant. The normality of the data distribution was assessed with Kolmogorov-Smirnov test. If the
variables were normally-distributed, data was expressed as mean ± SD. Differences among groups were analyzed with Analysis of variance (ANOVA) whereas between two groups were analyzed using LSD-t test. If not, data were expressed as interquartile range (median [P25, P75], P25 and P75 represent the 25% and 75% percentiles of data distribution, respectively. Differences among groups were analyzed with Welch t test and multiple comparisons were performed using Dunnet’s T3. Spearman correlation and multivariate logistic regression analysis were used to analyze associations between indices and EDKD. Spearman correlation was also used to analyze associations among indices. Binary logistic regression was performed to analyze the risk factors of EDKD and to establish diagnostic model of combined indices. Fisher Z conversion was performed on C1q, CysC and NGAL to avoid the influence caused by the difference of magnitudes among C1q, CysC and NGAL. The Z-scores were automatically calculated by MedCalc software. After Fisher Z conversion of C1q, CysC and NGAL, the risk of their Z-scores with EDKD occurrence was analyzed by binary logistic regression. ROC analysis was constructed to assess the diagnostic performance of the various indices. The Area Under Curve (AUC) was calculated. The statistical comparison of AUC was performed by DeLong test. For analysis of diagnostic value of more than one index, the ROC curve analysis was conducted using the predicted probability value (calculated by the binary logistic regression equation). The cutoff point was determined as the corresponding index level or predicted probability value with the largest Youden Index (YI; YI=sensitivity+specificity-1). ROC Curve Power Analysis was performed by
PASS11.0 software (NCCS Software, Kaysville, USA) for each index and combination.

The power was computed (power=1-beta).
3. Results

3.1. Characteristics of subjects

The clinical characteristics of SDM group, EDKD group and healthy controls were listed in Table I. Among the T2DM patients, there were 136 male patients with the average age of 58.5±11.6 (range from 22-72) years old and 109 female patients with the average age of 60.1±11.8 (range from 27-75) years old. In addition, there were 120 male and 181 female subjects in the control group, and their age ranged from 19-75 years old, with the average of 46.1±13.3 years old. Statistically significant difference was found in gender and age among the three groups (P<0.05). Kolmogorov-Smirnov test showed that except for SUr, all other serum indices were not normally-distributed. Significant differences were found in SUr, SCr, C1q, CysC and NGAL levels among the three groups (P<0.01). Additionally, Dunnet’s T3 showed that SUr, SCr, C1q and NGAL levels in EDKD group was significantly higher than that in other two groups (P<0.01).

3.2. Correlation analysis of EDKD with new serum indices

To determine the correlation between the observed laboratory indicators and EDKD, we next analyzed the association of EDKD with newly used serum indices including C1q, CysC, or NGAL, respectively. The result showed that EDKD was significantly related to C1q, CysC, or NGAL and the correlation was strongest for CysC while weakest for NGAL (Table II). Partial correlation analysis showed that the correlation still exists after adjustment using UACR, eGFR, age, gender, SUr and SCr as control variables. Risk factor analysis showed that the Odds Ratio (OR) was in the order
of CysC > C1q > NGAL, and all the three indices were risk factors for EDKD, before or after adjustment with age, SUr and SCr (Table III). Subsequently, we analyzed the correlation of C1q, CysC, or NGAL with classic renal function indices, including UACR, eGFR, SUr and SCr. These was no significant correlation of C1q with SCr or SUr (P > 0.05; Table IV), and correlation the of CysC or NGAL with the four classic renal function indicators was statistically significant (all P < 0.05; Table IV).

3.3. ROC curves of serum indices

ROC analysis showed that the diagnostic performance (indicated as AUC) of these indices was in the order of CysC (0.813)> C1q (0.797)> NGAL (0.770)> SCr (0.681)> SUr (0.650) (Table V and Figure 1A). Statistical analysis showed that these was no significant difference in diagnostic performance between SUr and SCr (z=0.893, p > 0.05), or among C1q, CysC, and NGAL (p > 0.05). However, the diagnostic performance of C1q, CysC, and NGAL were significantly higher than that of the former two (SUr and SCr) (p < 0.05).

Although SCr showed relative smaller AUC, it had the highest specificity among the five indices. Therefore, the diagnostic performance of SCr in combination with other indices was also evaluated (Table V and Figure 1B). The AUC of the combined indices was in the order of C1q+CysC (0.896)> C1q+NGAL (0.866)> CysC+NGAL (0.839)> SCr+C1q (0.834)> SCr+CysC (0.820)> SCr+NGAL (0.779). Statistical analysis showed that the diagnostic performance of C1q+CysC was significantly higher
than that of CysC+NGAL, SCr+C1q, SCr+ CysC, and SCr+NGAL (p<0.05). Moreover, C1q+NGAL had the second highest diagnostic performance and its diagnostic performance was significantly higher than that of SCr+NGAL (p<0.05).

Next we analyzed the diagnostic performance of the combination of these serum indices. The AUC of C1q+CysC+NGAL is 0.909 (Table V and Figure 1C), indicating its high diagnostic performance.

Finally we tested the diagnostic value of C1q+NGAL+CysC+SCr. Although the AUC of C1q+NGAL+CysC+SCr (0.912) was the highest, it showed no statistical difference when compared with C1q+CysC+NGAL (0.909), SCr+ C1q+NGAL (0.898), or C1q+CysC+SCr (0.876) (p>0.05; Table V and Figure 1D). Moreover, the AUC of CysC+C1q+NGAL+SCr (AUC=0.912) was only slightly increased than CysC+C1q+NGAL, without significant difference (z=1.616, p>0.05).

Together, CysC, C1q and NGAL showed similar diagnostic performance, and diagnostic performance of combination of any two indices was better than that of any single one (p<0.05). According to the final sample sizes (positive 109 and negative 437), ROC Curve Power Analysis showed that power of the study (0.9912-1.0000) of any combination of C1q, NGAL and CysC was higher than that of single index.

4. Discussion

In this study, we evaluated the diagnostic performance of serum C1q, CysC and NGAL levels for EDKD alone and in combination, providing a basis for the early diagnosis of DKD clinically. The results of this study showed that the diagnostic performance of C1q,
CysC and NGAL for EDKD was similar and better than SCr. When considering the cost of detection, the combination of C1q and CysC has the best diagnostic performance for EDKD. DKD is a common complication of diabetes. Early diagnosis of DKD and active early intervention can not only prevent or delay the onset of end-stage renal disease, but also may reverse renal damage. C1q, CysC and NGAL, especially the latter two, are emerging indicators for renal function evaluation [30-32]. In particular, CysC has been written into the KIDGO (2012) CKD Clinical Application Guideline, which is recommended worldwide [7]. Although studies on various types of kidney diseases have shown that C1q, CysC and NGAL have good clinical application value individually [33-35], their synergistic effects are still unknown.

Complement C1q is closely related to immune-related diseases and has long been used as an immune-related indicator. Serum C1q also has important clinical value in assisting diagnosis and treatment of diseases such as autoimmune nephropathy [24]. Studies reported that there was a large number of C1q and other complement component deposition in isolated pancreatic tissue of patients with T2DM [36, 37], suggesting that changes in blood C1q levels may be associated with the onset of T2DM.

In addition, C1q deposition in the glomerular portal area is positively correlated with DKD severity [38]. NGAL is a newly identified adipokine that is highly expressed in cells from various pathological tissues with abnormal metabolism [39, 40]. Many studies have shown that NGAL has a rapid elevation at blood levels during kidney injury, and its sensitivity for ischemic or nephrotoxic AKI is significantly higher than
traditional kidney injury markers such as SCr and SUr [35, 41, 42]. Since its recommendation by the US FDA as a marker of renal function in 2002, CysC has been widely used in clinical applications because it is not affected by factors such as age, gender, and body weight inflammation. In this study, we found that serum levels of C1q, NGAL and CysC were significantly higher in patients with T2DM compared with those in healthy controls, as well as in EDKD patients compared with SDM patients (Table I). Moreover, C1q, CysC, and NGAL were all significantly correlated with EDKD and the correlation was strongest for CysC while weakest for NGAL (Table II). These results indicate that these three indices may be used as diagnostic markers for EDKD.

Analyzing of risk factors found that C1q, NGAL and CysC were all risk factors for EDKD (Table III). There was significant correlation between CysC or NGAL and classic renal function indicators (UACR, eGFR, SUr and SCr) (Table IV). ROC analysis also demonstrated that the diagnostic performance of C1q, NGAL and CysC were significantly higher than SCr, whereas that of C1q, NGAL and CysC was similar (Table V and Figure 1). These data indicate that serum level of C1q, NGAL and CysC is efficient for EDKD diagnosis. In addition, the sensitivity of C1q was highest (80.73%) and the AUC of C1q (0.797) and CysC (0.813) were comparable. Although the AUC of SCr was relatively low, its specificity was the highest among all single indices. Moreover, SCr is a widely-accepted and generally used biomarker for impaired renal function [43, 44]. Thus, SCr may be used as a clinical biomarker for assessing renal function of EDKD.
According to the clinician's diagnostic and therapeutic habits, we analyzed the diagnostic performance of combinations of two, three and four combinations. In the combination of two indices, C1q+CysC, C1q+NGAL and CysC+NGAL showed significantly higher AUC when compared with any single index (Table V), suggesting that they have higher value in the diagnosis of EDKD. Since the C1q+CysC combination had the highest AUC, we suggest that this combination has the most application potential among all 2-index groups. In the combination of three indices, we found that CysC+C1q+NGAL and CysC+C1q+NGAL+SCr had the best diagnostic performance, however, both combinations failed to show significantly increase in AUC compared with C1q+CysC (Table V). It is well known that adding diagnostic tests will increase the diagnostic performances; however, the cost will also increase. The results of this study showed that the diagnostic performance of C1q+CysC was slightly lower than that of C1q+NGAL+CysC+SCr. However, if the economic factors are taken into account, the combination of C1q+CysC may be more cost effective than C1q+NGAL+CysC+SCr. Taking account into all aspects (such as testing cost and specificity), we believe that C1q+CysC combination might be the best one for EDKD diagnosis. However, further studies are still needed to investigate this.

Additionally, there were differences in age and gender among EDKD, SDM and healthy controls. One reason may be that healthy controls were randomly sampled. Another reason is the gradual damaging course from SDM to EDKD, resulting in older age for EDKD patients than SMD patients. However, there was no difference in gender
between SDM and EDKD groups, indicating that these two groups were comparable. Although studies have shown that serum CysC levels are not affected by age and sex [12, 18, 21], in theory, this difference is likely to induce bias in results. Therefore, further studies with age and gender matched subjects are needed to verify our results.

This study has the following limitations: First, the differences in age and gender among EDKD, SDM and healthy controls may induce bias in the results of this study. Secondly, eGFR (≥ 60 mL/min/1.73 m²) was used as the cutoff point for EDKD patient selection, however, G1 and G2 phases of CKD may also be included by this criterion. Thirdly, eGFR was calculated by CysC. The experimental errors for detecting CysC may lead to false diagnosis of EDKD, which will affect the accuracy of the results. Further studies with age and gender matched subjects and more strict inclusion criteria are warranted.
5. Conclusions

In summary, our study shows that C1q, NGAL and CysC have good performance for diagnosing EDKD, which may be further improved by the C1q+CysC combination. In contrast, it is difficult to differentiate the early damage of renal function in patients with T2DM by the traditional renal function test index-SCr and/or its resulting eGFR. Therefore, the indicators of C1q, NGAL, CysC or the combination of C1q+CysC may enable the early diagnosis and early treatment of EDKD, delaying the development of DM. Our findings may provide experimental evidence for the development of more effective diagnostic markers for EDKD.
Acknowledgements: None.

Funding: This work was supported by the sub-topics of National Basic Research Program of China (973 Program; 2015CB755402-043) and Science and Technology Department of Sichuan Province (2015SZ0117).

Declaration of interest: None.


Figure 1. ROC curves for individual or combined indicators in the diagnosis for EDKD.

The diagnostic efficiency of each observed indicators, in terms of sensitivity and specificity, are presented after ROC curve analysis. (A) single indicator; (B) two indicators combination; (C) three indicators combination; (D) four indicators combination.
Table IV. Correlation of C1q, CysC, or NGAL with classic renal function indices 
\((r, 95\% CI)\).

<table>
<thead>
<tr>
<th></th>
<th>UACR</th>
<th>eGFR</th>
<th>SUr</th>
<th>SCr</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1q</td>
<td>0.308* (0.229, 0.383)</td>
<td>-0.188* (-0.267, -0.099)</td>
<td>0.084 (0.003, 0.167)</td>
<td>0.045 (-0.045, 0.133)</td>
</tr>
<tr>
<td>CysC</td>
<td>0.287* (0.201, 0.367)</td>
<td>-0.986* (-0.998, -0.968)</td>
<td>0.378* (0.304, 0.446)</td>
<td>0.620* (0.562, 0.675)</td>
</tr>
<tr>
<td>NGAL</td>
<td>0.263* (0.183, 0.342)</td>
<td>-0.454* (-0.528, -0.389)</td>
<td>0.148* (0.059, 0.237)</td>
<td>0.344* (0.264, 0.420)</td>
</tr>
</tbody>
</table>

Note: Data was analyzed using Spearman correlation. *\(P<0.05\).
Table I. Clinical characteristics of patients in each group

<table>
<thead>
<tr>
<th>Groups</th>
<th>HC (n=301)</th>
<th>SDM (n=136)</th>
<th>EDKD (n=109)</th>
<th>$F$/$\chi^2$, adjusted $P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>47.0 (38.0, 54.0)</td>
<td>60.0 (52.0, 68.0)$^a$</td>
<td>65.0 (54.0, 69.0)$^{ab}$</td>
<td>74.86, &lt;0.001</td>
</tr>
<tr>
<td>Sex (Male/Female)</td>
<td>120/181</td>
<td>74/62</td>
<td>62/47</td>
<td>13.42, 0.001</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td>5.17 (4.88, 5.44)</td>
<td>8.94 (7.87, 11.89)$^a$</td>
<td>9.78 (7.88, 14.31)$^a$</td>
<td>320.23, &lt;0.001</td>
</tr>
<tr>
<td>SUr (mmol/L)</td>
<td>5.08±1.17</td>
<td>5.54±1.64$^a$</td>
<td>6.63±2.92$^{ab}$</td>
<td>30.903, &lt;0.001</td>
</tr>
<tr>
<td>SCr (μmol/L)</td>
<td>59.40 (51.70, 69.65)</td>
<td>59.10 (48.50, 70.57)</td>
<td>72.00 (57.75, 88.50)$^{ab}$</td>
<td>28.432, &lt;0.001</td>
</tr>
<tr>
<td>CysC (mg/L)</td>
<td>0.75 (0.67, 0.86)</td>
<td>0.84 (0.74, 0.99)$^a$</td>
<td>1.08 (0.90, 1.35)$^{ab}$</td>
<td>118.195, &lt;0.001</td>
</tr>
<tr>
<td>eGFR ( ml·min$^{-1}$·1.73 m$^{-2}$)</td>
<td>103.77 (91.98, 115.69)</td>
<td>123.80 (96.20, 160.67)$^a$</td>
<td>73.00 (60.69, 87.05)$^{ab}$</td>
<td>77.330, &lt;0.001</td>
</tr>
<tr>
<td>UACR ( mg/g)</td>
<td>9.40 (5.39, 14.14)</td>
<td>10.56 (6.07, 18.00)$^a$</td>
<td>80.59 (45.78, 152.84)$^{ab}$</td>
<td>326.436, &lt;0.001</td>
</tr>
<tr>
<td>C1q ( mg/L)</td>
<td>163.60 (147.45, 186.60)</td>
<td>176.90 (170.70, 183.75)$^a$</td>
<td>192.50 (184.80, 199.55)$^{ab}$</td>
<td>62.822, &lt;0.001</td>
</tr>
<tr>
<td>NGAL (μg/L)</td>
<td>104.20 (147.75, 186.60)</td>
<td>123.80 (96.20, 160.67)$^a$</td>
<td>163.00 (122.10, 223.05)$^{ab}$</td>
<td>56.044, &lt;0.001</td>
</tr>
</tbody>
</table>

Note: Data for SUr was expressed as mean ±SD while the others were expressed as M (P$_{25}$, P$_{75}$). $^a$ The difference in Sex was compared by chi-square test, and for SUr by One-way ANOVA, and for the others by Welch t test. $^b$P$<$0.0167, compared with HC group by LSD-t test for SUr and Dunnet’s T3 test for the others; $^{ab}$P$<$0.0167, compared with SDM group by LSD-t test for SUr and Dunnet’s T3 test for the others. 
HC, healthy control; SDM, Single T2DM cases; EDKD, early diabetic kidney disease.
Table II. Correlation analysis of C1q, CysC, or NGAL with EDKD occurrence.

<table>
<thead>
<tr>
<th>Index</th>
<th>Spearman correlation</th>
<th>Partial correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r (95%CI)</td>
<td>t</td>
</tr>
<tr>
<td>C1q</td>
<td>0.412 (0.340, 0.477)</td>
<td>10.219</td>
</tr>
<tr>
<td>CysC</td>
<td>0.433 (0.351, 0.508)</td>
<td>14.467</td>
</tr>
<tr>
<td>NGAL</td>
<td>0.374 (0.296, 0.447)</td>
<td>10.061</td>
</tr>
</tbody>
</table>

Note: The r represents Spearman's correlation coefficient of the observed indicator with EDKD occurrence. The r_p represents partial correlation coefficient of the observed indicator with EDKD occurrence, which was analyzed by multivariate logistic regression with adjusted variables, including UACR, eGFR, age, sex, SUr and SCr.
Table III. Risk factors for EDKD.

<table>
<thead>
<tr>
<th>Index</th>
<th>Before adjustment</th>
<th>After adjustment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR (95%CI)</td>
<td>Wald $x^2$</td>
</tr>
<tr>
<td>C1q</td>
<td>4.336 (2.996, 6.277)</td>
<td>60.456</td>
</tr>
<tr>
<td>CysC</td>
<td>4.761 (3.448, 6.576)</td>
<td>89.737</td>
</tr>
<tr>
<td>NGAL</td>
<td>3.341 (2.426, 4.601)</td>
<td>54.627</td>
</tr>
</tbody>
</table>

Note: Using Logistic regression analysis, the adjusted variables include age, sex, SUr and SCr.
Table V. The sensitivity and specificity of serum biomarkers for EDKD.

<table>
<thead>
<tr>
<th>Index</th>
<th>Cut-off* Value</th>
<th>AUC</th>
<th>AUC(95% CI)</th>
<th>P</th>
<th>Se (%)</th>
<th>95% CI</th>
<th>Sp (%)</th>
<th>95% CI</th>
<th>YI</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUr</td>
<td>5.34</td>
<td>0.650</td>
<td>0.608 - 0.690</td>
<td>&lt;0.001</td>
<td>67.89</td>
<td>63.79 - 71.79</td>
<td>58.81</td>
<td>54.55 - 62.97</td>
<td>0.267</td>
</tr>
<tr>
<td>SCR</td>
<td>70.6</td>
<td>0.681</td>
<td>0.640 - 0.720</td>
<td>&lt;0.001</td>
<td>55.05</td>
<td>50.77 - 59.28</td>
<td>76.20</td>
<td>72.40 - 79.71</td>
<td>0.313</td>
</tr>
<tr>
<td>NGAL</td>
<td>134.7</td>
<td>0.770</td>
<td>0.732 - 0.805</td>
<td>&lt;0.001</td>
<td>69.72</td>
<td>65.68 - 73.55</td>
<td>73.92</td>
<td>70.02 - 77.56</td>
<td>0.436</td>
</tr>
<tr>
<td>C1q</td>
<td>184.2</td>
<td>0.797</td>
<td>0.761 - 0.830</td>
<td>&lt;0.001</td>
<td>80.73</td>
<td>77.17 - 83.96</td>
<td>70.25</td>
<td>66.22 - 74.06</td>
<td>0.510</td>
</tr>
<tr>
<td>CysC</td>
<td>0.89</td>
<td>0.813</td>
<td>0.777 - 0.845</td>
<td>&lt;0.001</td>
<td>77.06</td>
<td>73.30 - 80.52</td>
<td>75.06</td>
<td>71.21 - 78.64</td>
<td>0.521</td>
</tr>
<tr>
<td>SCR+NGAL</td>
<td>-</td>
<td>0.779</td>
<td>0.742 - 0.814</td>
<td>&lt;0.001</td>
<td>64.22</td>
<td>60.04 - 68.25</td>
<td>79.18</td>
<td>75.53 - 82.51</td>
<td>0.434</td>
</tr>
<tr>
<td>SCR+CysC</td>
<td>-</td>
<td>0.820</td>
<td>0.785 - 0.851</td>
<td>&lt;0.001</td>
<td>77.98</td>
<td>74.27 - 81.39</td>
<td>73.68</td>
<td>69.77 - 77.33</td>
<td>0.517</td>
</tr>
<tr>
<td>SCR+C1q</td>
<td>-</td>
<td>0.834</td>
<td>0.800 - 0.864</td>
<td>&lt;0.001</td>
<td>71.56</td>
<td>67.57 - 75.31</td>
<td>83.03</td>
<td>79.61 - 86.08</td>
<td>0.546</td>
</tr>
<tr>
<td>CysC+NGAL</td>
<td>-</td>
<td>0.839</td>
<td>0.806 - 0.869</td>
<td>&lt;0.001</td>
<td>81.65</td>
<td>78.05 - 84.73</td>
<td>73.68</td>
<td>69.77 - 77.33</td>
<td>0.553</td>
</tr>
<tr>
<td>C1q+NGAL</td>
<td>-</td>
<td>0.866</td>
<td>0.834 - 0.893</td>
<td>&lt;0.001</td>
<td>75.23</td>
<td>71.39 - 78.80</td>
<td>81.46</td>
<td>77.94 - 84.63</td>
<td>0.567</td>
</tr>
<tr>
<td>C1q+CysC</td>
<td>-</td>
<td>0.896</td>
<td>0.867 - 0.920</td>
<td>&lt;0.001</td>
<td>77.98</td>
<td>74.27 - 81.39</td>
<td>89.47</td>
<td>86.59 - 91.92</td>
<td>0.675</td>
</tr>
<tr>
<td>SCR+NGAL+CysC</td>
<td>-</td>
<td>0.845</td>
<td>0.811 - 0.874</td>
<td>&lt;0.001</td>
<td>79.82</td>
<td>76.20 - 83.11</td>
<td>75.51</td>
<td>71.68 - 79.06</td>
<td>0.553</td>
</tr>
<tr>
<td>SCR+C1q+NGAL</td>
<td>-</td>
<td>0.876</td>
<td>0.845 - 0.902</td>
<td>&lt;0.001</td>
<td>87.16</td>
<td>84.06 - 89.85</td>
<td>71.85</td>
<td>67.87 - 75.59</td>
<td>0.590</td>
</tr>
<tr>
<td>C1q+CysC+SCR</td>
<td>-</td>
<td>0.898</td>
<td>0.870 - 0.922</td>
<td>&lt;0.001</td>
<td>83.49</td>
<td>80.10 - 86.51</td>
<td>83.30</td>
<td>79.90 - 86.33</td>
<td>0.668</td>
</tr>
<tr>
<td>C1q+CysC+NGAL</td>
<td>-</td>
<td>0.909</td>
<td>0.882 - 0.932</td>
<td>&lt;0.001</td>
<td>88.99</td>
<td>86.06 - 91.49</td>
<td>80.32</td>
<td>76.73 - 83.57</td>
<td>0.693</td>
</tr>
<tr>
<td>C1q+NGAL+CysC+SCR</td>
<td>-</td>
<td>0.912</td>
<td>0.885 - 0.934</td>
<td>&lt;0.001</td>
<td>87.16</td>
<td>84.06 - 89.85</td>
<td>83.98</td>
<td>80.63 - 86.96</td>
<td>0.711</td>
</tr>
</tbody>
</table>

Note: AUC, area under curve; YI, Youden Index; Se, Sensitivity; Sp, Specificity. * The units of Sur, SCR, NGAL, C1q and CysC are mmol/L, μmol/L, mg/L, mg/L and mg/L, respectively.