https://doi.org/10.48047/AFJBS.6.Si3.2024.1250-1259

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Early detection of diabetic nephropathy using Serum C4d complement in patients with type 2 diabetes mellitus.

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Article Info

Volume 6, Issue Si3, May 2024

Received: 09 March 2024

Accepted: 19 May 2024

Published: 15 Jun 2024

doi:10.48047/AFJBS.6.Si3.2024.1250-1259

Abstract

Background: Diabetic nephropathy (DN) has been associated with increased renal expression of complement factors C3, C4, and C9, as well as increased expression of C1q, C1s, and C1r, which suggest a role for the classical complement pathway.

Objective: To investigate serum C4d as a biomarker for diabetic nephropathy in T2 diabetes.

Materials and methods: we enrolled 120 patients with Type 2 diabetes, along with 60 healthy control subjects. Kidney function tests, albumin/creatinine ratio, serum HbA1c& serum C4d were measured in all candidates.

Results: Creatinine was significantly higher and eGFR was significantly lower in the group with diabetes as compared to controls (P <0.001), Serum C4d was significantly higher in the group with diabetes.There was a significant positive correlation between C4d on one hand, and HbA1c, creatinine, albuminuria, and progression of DKD stage on the other hand.Multivariable regression showed that age, HbA1c and C4d level were independent predictors of DKD and its stages. C4d, at a cut off value > 1 predicted DM with 80% specificity and 38% sensitivity(P 0.02).

Conclusion: The complement fragment C4d level is strongly associated with diabetic nephropathy and its stages. C4d is significantly higher in patients with diabetic nephropathy, and it is positively correlated with creatinine, decline of GFR and proteinuria. Also, its level increases with the progression of DKD stage. C4d is a specific rather than sensitive tool for early detection of DKD.

Keywords: diabetic nephropathy, C4d, complement system.

INTRODUCTION

Diabetes has a significant influence on one's health and is associated with significant individual and social consequences. (1,2)

More than 1 million deaths were attributed to diabetes and its complications.(3)

The UK Prospective Diabetes Study (UKPDS) of 1998 and the Steno-2 Study are both notable research endeavors in the field of diabetes. (4)Research has shown that persistent hyperglycemia is a contributor to complications in diabetes. The extent of tissue damage in individuals with diabetes is influenced by both genetic susceptibility factors and accelerating variables such as hypertension and dyslipidemia.

An expanding body of clinical and experimental evidence indicates an association between the complement system, complement regulatory proteins and the pathophysiology of diabetes complications. (5,6)New findings suggest that the complement system plays a role in various aspects of cardiometabolic sequalae.This involves disturbances in the metabolism of adipose tissue, subtle localized inflammation, increased activity of adhesion molecules and the presence of proinflammatory cytokines within endothelial cells, resulting in both endothelial dysfunction as well as insulin resistance. (7,8)Previous studies have established that people with diabetic nephropathy had significantly higher urine C4blevels than subjects without the condition, which may be a factor in delaying the progression of DN to end-stagerenal disease (ESRD). As per these findings, C4b may be used as a non-intrusive biomarker for the early diagnosis of DN(9).

It's been proven that by the time microalbuminuria presents clinically, severe structural abnormalities in the glomerular basement membrane may already have occurred. (10). Microalbuminuria, when considered as a whole, may be more of a diagnostic sign than a tool for predicting DN. As a result, as per our work and other published studies, using the measurement of C4d in bloodshould be considered among alternative biomarkers for the early prediction of DN.

The goal of this study is to look at the role of complement activation using plasma C4d in the early detection of DN in people with type 2 diabetes.

PATIENTS AND METHODS

We recruited 120 patients diagnosed with T2DM from internal medicine wards or internal medicine & endocrinology clinics, and 60 healthy control subjects of the same age and gender to conduct a cohort study.

Any patient who was having type 2 diabetes was eligible for inclusion in the current study, while patients with non-diabetic kidney disease, autoimmune disease, malignancy and chronic infectious disease were excluded.

All participants signed an informed consentafter explaining the study procedure, presumed risks & benefits. All patients were subjected to complete history taking, systematic clinical examination, blood pressure measurement (hypertension was diagnosed in our study if it is ≥ 140/90 according to ADA 2020), waist circumference and pinprick testing for peripheral neuropathy.Pin prick testing was done by a pin applied proximally to the big toenail, (0 if normal, 1 if peculiar). A Fundus examination was done to screen for diabetic retinopathy.

Laboratory tests as HbA1C, AST, ALT and fasting lipid profile.As regards HbA1c, it was done using the immunoturbidmetry technique, and normal level is considered below 5.7% percent, prediabetes ranges from 5.7% to 6.4% and diabetes from 6.5% and above (11).Dyslipidemia is

considered if serum cholesterol is above 200 mg/dL, triglycerides above 150 mg/dL (HDL) cholesterol below 60 mg/dL and (LDL) cholesterol above 100 mg/dL (12).

Albumin creatinine ratio was measured by dividing urinary albumin in mg/L by urinary creatinine in gm/L in spot urine sample,where normo-albuminuria with ACR: < 30 mg/ gm creatinine, moderately increased albuminuria with ACR 30 mg-300 mg/gm creatinine and severely increased albuminuria (macro-albuminuria) with ACR>300 mg/ gm creatinine were defined (13).

Estimated GFR was calculated using the CKD-EPI creatinine equation (14)
GFR = 141 x min (Scr/κ,1) α x max (Scr/κ, 1)-1.209 x 0.993Age x 1.018 [if female] x 1.159 [if black]
Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1(15). Estimated GFR CKD-EPI was measured by mobile application.

Complement fragment 4d:
Serum human complement fragment (C4d) testing was done to all subjects. The kit uses enzyme-linked immunosorbent assay (ELISA) to assay the level of Human Complement Fragment 4d.

Ethical approval and consent to participate: study protocol and informed consent were submitted for Institutional Review Board and Ethical Committee at the internal medicine department of XXX and approval was granted on 19.2.2019.

Statistical analysis:
The analysis was conducted utilizing MedCalc Statistical Software version 19.2 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). The assessment of normal distribution for continuous data was carried out through the D'Agostino-Pearson test. It was observed that normal distribution was not evident in all variables except for age, consequently leading to the application of non-parametric tests.For comparison, the Mann-Whitney test was utilised. Ordinal data was subjected to the Chi-squared test.

RESULTS

A full set analysis was formed of 120 diabetic patients along with 60 gender matched healthy controls. Creatinine was significantly higher and eGFR was significantly lower in the group with diabetes as compared to controls (P <0.0001).C4d was significantly higher in the group with diabetes with (P < 0.009) table 1.

Table 1: Comparison of Laboratory Results between the study group and the control group

	Diabetes Group		**Control Group**		
	Range	Mean ± SD	Range	Mean ± SD	P value
Age	41 to 82	58.9 ± 8.6	21 to 71	43.1 ± 12.4	<0.001’
Females (n, %)	73	60.8%	44	73.3%	0.097*

Creatinine (mg/dL)	0.55 to 9	1.82 ± 1.76	0.45 to 1.0	0.76 ± 0.11	<0.0001
eGFR (mL/min/1.73 m2)	4.34 to 134.9	70.8 ± 28.2	90 to 120	106 ± 10.39	<0.0001
C4d	0.4 to 19.5	1.673 ± 2.94	0.3 to 1.8	0.75 ± 0.311	0.009
HbA1c (Normally: 4-5.6%)	6 to 15.3	8.7 ± 1.81	.	.	NA
Albuminuria	0 to 1477	145 ± 253.4	.	.	NA
‘ independent T-test, Mann Whitney U test, *Chi2 test.					

Ninety seven patients were diagnosed with DKD, 60 of which had both albuminuria greater than 30 by albumin creatinine ratio and decreased eGFR < 90. Eleven of the DKD patients, however, had a normal eGFR with albuminuria and 26 had no albuminuria but had decline of eGFR. In the diabetic group the number of patients with decreased eGFR is 86 (71%). Sixteen patients (13.3% of the study group) had macroalbuminuria and 55 patients (45.8% of the study group) had microalbuminuria.

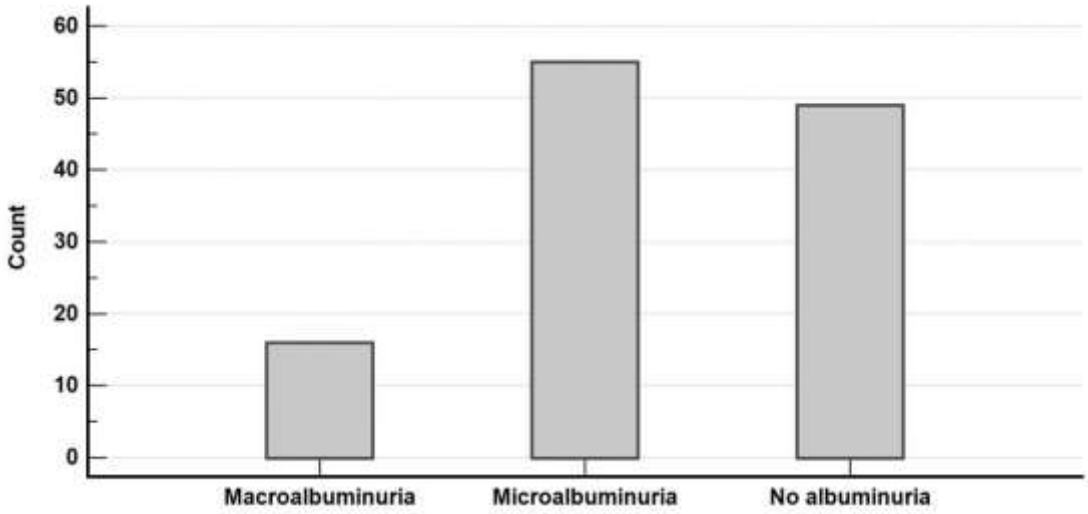

Figure 1: counts of patients included regarding albuminuria: Sixteen patients (13.3% of the study group) had macroalbuminuria and 55 patients (45.8% of the study group) had microalbuminuria.

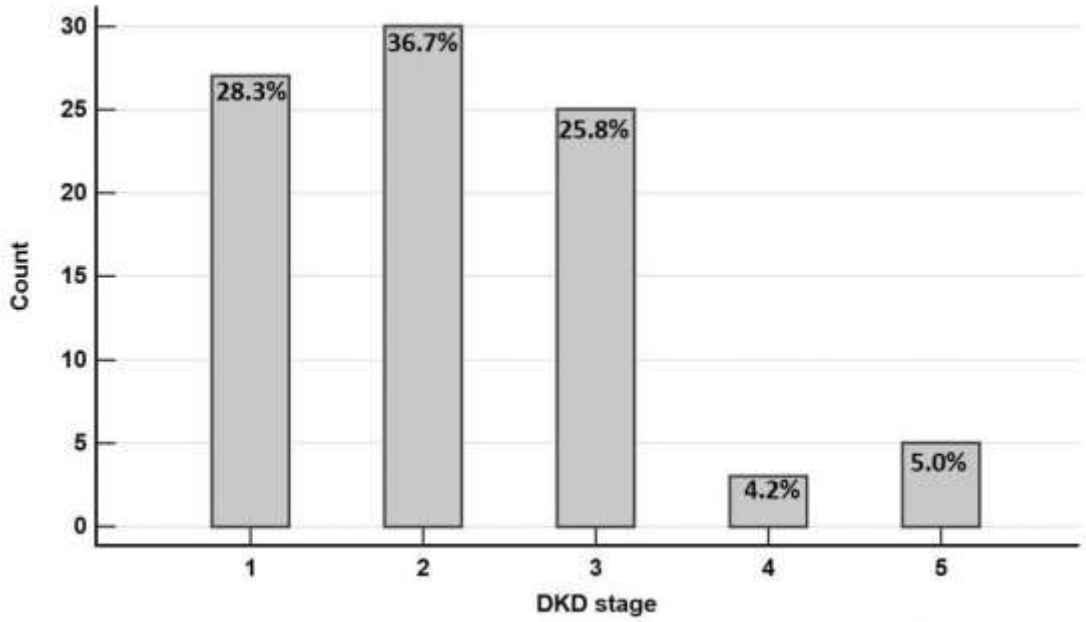

Figure 2: frequency of DKD stages in patients with diabetes

Univariate Regression analysis showed that there is positive relationship between C4d, creatinine, albuminuria, stage of DN, HbA1c, and duration of diabetes. While C4d was negatively correlated to eGFR. Table 2.

Table 2: univariate linear regression model showing relationship of C4d versus various variables among diabetic patients.

Variable	Coefficient	Std. Error	95% CI	t	P
Albuminuria	0.004	0.001	0.0023 to 0.0062	4.336	<0.0001
Creatinine	0.556	0.111	0.3360 to 0.7758	5.005	<0.0001
DKD stage	0.745	0.106	0.5346 to 0.9555	7.010	<0.0001
eGFR	-0.017	0.004	0.009715 to 0.02453	4.577	<0.0001
HbA1c	0.183	0.030	0.1229 to 0.2423	6.055	<0.0001
Duration of DM	0.133	0.027	0.07986 to 0.1870	4.932	<0.0001

All potiential variables that can affect diabetic kidney disease which are age, gender, Hba1c, dyslipidemia, elevated liver enzymes, retinopathy, neuropathy and duration of diabetes, were entered together with the C4d level into a multiple regression model as independent predictors of DKD. DKD was expressed in terms of the 5 DKD stages. Multivariable regression analysis showed that age, serum C4d level, and HbA1c were independent predictors of DKD and progression to ESRD. Table 3.

Table 3: Multivariable linear regression model showing significant variables, that are independent predictors of DKD progression through the 5 stages.

Independent variables	Coefficient	Std. Error	t	P value
Age	0.04689	0.007577	6.189	<0.0001
C4d	0.06272	0.02825	2.22	0.028

HbA1c	0.09005	0.04531	1.988	0.049

Reciever Operating Characteristic (ROC) curve analysis showed that C4d can significantly predict GFR <90 ml/kg/min, and nephropathy using cutoff point >1 with sensitivity 38.37 %, specificity 79.79 % and sensitivity 37.11 %, specificity 80.72% respectively. It was NOT significant as a predictor of proteinuria.

Table 4: ROC curve analysis testing of C4d against various outcomes

Variables	Cut toff	Sensitivity %	Specificity %	AUC	P value
Proteinuria	>1.5	22.54	94.5	0.525	0.5763
GFR decline	>1	38.37	79.79	0.599	0.0184
Nephropathy	>1	37.11	80.72	0.597	0.0213

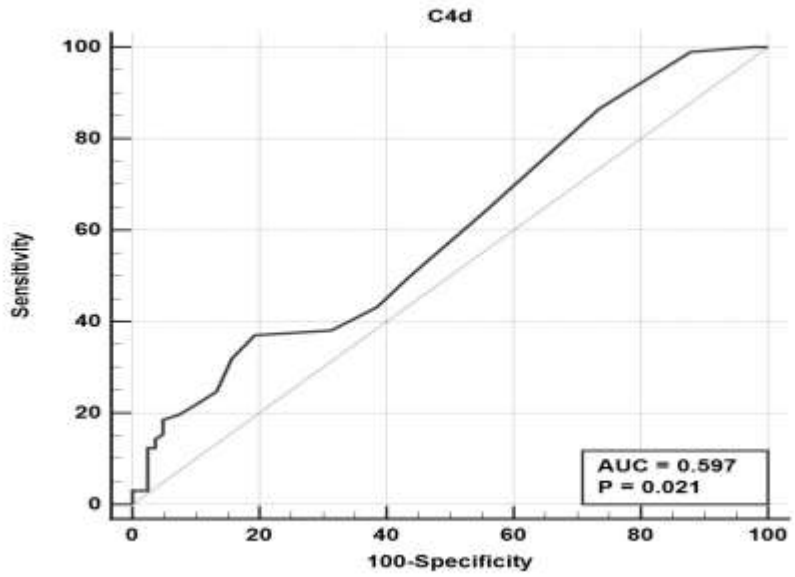

Figure 3 Receiver Operating Characteristic (ROC) curve analysis of C4d as a marker of nephropathy(proteinuria and/or GFR decline).

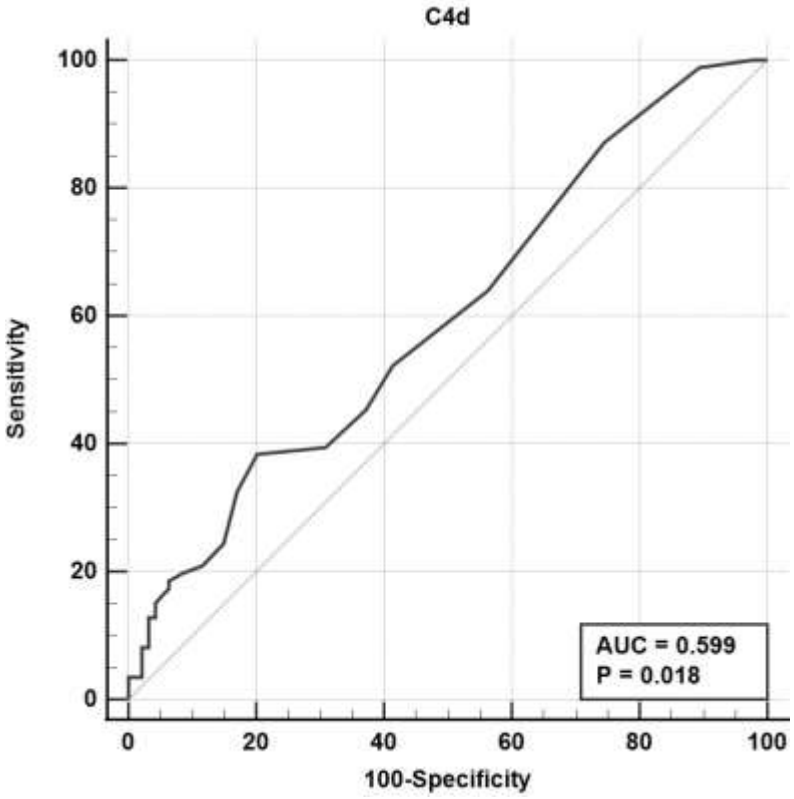

Figure 4 Receiver Operating Characteristic (ROC) curve analysis of C4d as a marker of GFR decline.

DISCUSSION

Diabetic kidney disease (DKD) normally progresses over period of time and eventually results in end-stage kidney disease. A developing pathogenesis of inception as well as also progression of DKD is complement system activation. We steered a cross section analytical study to assess the complement activation using plasma C4d in early recognition of DN in type 2 diabetics. To the best of our understanding, this study represents the initial identification of an association between serum C4d levels and the extent of decline in renal function.

In the current study we found that renal functions were significantly impaired among diabetics compared to heathy population. The incidence of decreased eGFR, microalbuminuria, and macroalbuminuria among the included diabetics were 71%, 45.8%, and 13.3% correspondingly.

These findings were higher than the rates reported by Kene et al., and Nata et al., 43% and 39.2% respectively. We believe that this difference was principally due to the larger sample size of later mentioned studies (16, 17).

We found that serum C4d was significantly higher in the group with diabetes versus healthy participants. It was significantly correlated with albuminuria, and stage of DKD. Our findings are coherent with Pascal et al., who discovered that subjects with diabetes had much higher rates of

C4d deposits in the glomeruli than did the non-diabetic cases. C4d deposits were found in 38%, 48%, and 22% of the cases, respectively, in the glomeruli and glomerular hili, as well as arterioles (18).

Li, et al., asserted 7 distinct complement components (C1q, MBL, Bb, C4d, C3a, C5a, and sC5b-9) were measured in blood plasma and urine to demonstrate complement activation in diabetic nephropathy. Patients with DKD had considerably greater levels of these components than DM patients without renal involvement (19).

Our results support the findings reported by Wlazlo et al., who found that baseline levels of C3 were originally associated with incident T2DM. as well, plasma C3 levels were originally linked to estimated insulin resistance in muscle, liver, and adipocytes, and glucose tolerance (20).

Urinary complement component levels and proteinuria (sC5b-9, C4a, factor Bb, MASP-1, and C1q) in diabetic patients were found to be significantly correlated by Pelletier et al. Additionally, an association was discovered between complement activation and the loss in renal function as well as a strong correspondence with the control of diabetes (21).
Our data showed that C4d can pointedly predict eGFR <90 ml/kg/min, and nephropathy using cutoff point >1 with sensitivity 38.37 %, specificity 79.79 % and sensitivity 37.11 %, specificity 80.72% respectively. Our study results signify that serum C4d is higher along with the progression of nephropathy. Therefore it functions as one of the biomarkers that can detect early diabetic nephropathy changes.
C4d as a marker failed to predict proteinuria per se, but it was specific for GFR decline, and progression through DKD stages. This correlation with proteinuria stands in opposition with Duan et al , who found that serum C4 levels showed positive associations with proteinuria, the rate of the progression to ESRD and doubling serum creatinine (22).
The main strength of the current study is the innovation of measuring serum C4d compared to other studies that assessed Cd4 deposits in renal tissues and urinary complements. We faced few restraints in terms of relatively small sample size compared to above mentioned cross section studies, being a single institutional study, which limits the generalizability of the study results. Further studies are still commanded to evaluate the role of serum C4d as a predictor for diabetic nephropathy.
Accordingly, our conclusion is: The complement fragment C4d level is associated with diabetic nephropathy and its stages. C4d is significantly higher in patients with diabetic nephropathy, and it positively shows a relationship with creatinine, decline of GFR and proteinuria. Also, its level increases with the progression of DKD stage. C4d is a specific rather than sensitive tool for early detection of DKD.

CONCLUSION

The complement fragment C4d level is strongly associated with diabetic nephropathy and its stages. C4d is significantly higher in patients with diabetic nephropathy, and it is positively correlated with creatinine, decline of GFR and proteinuria. Also, its level increases with the progression of DKD stage. C4d is a specific rather than sensitive tool for early detection of DKD.

Acknowledgments:

We would like to acknowledge our great Kasr Al Ainy Hospital and its workers, nurses and staff members for all the support and help in this study and throughout our careers.

Authors' contribution:

AA and AH analyzed and interpreted the patient's data. AA collected the data. UA wrote the statistics of the manuscript. SF was a major contributor in writing the manuscript. AE and AS suggested the objective of the work, supervised the work and revised the results and manuscript. All authors read and approved the final manuscript.

Funding:

Authors received no funding for this study.

Declarations:

Ethical approval and consent to participate: study protocol and informed consent were submitted for Institutional Review Board and Ethical Committee at the internal medicine department of Cairo University and approval was granted on 19.2.2019.

Consent for publication:

Oral and written informed consents were obtained from the patients or from the eligible relatives.

Competing interest:

The authors declare that they have no competing interests.

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