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Expression and clinical association of MFG-E8 and TAM receptors in diabetic patients with different stages of microvascular complication

An experimental study

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Abstract

Background: Diabetic nephropathy (DN) is a major microvascular complication of diabetes mellitus that leads to end-stage renal disease. Hyperglycemia triggers apoptosis and kidney damage. Milk fat globule-epidermal growth factor 8 (MFG-E8) and TAM receptor tyrosine kinases, Tyro3, Axl, and Mer, are phagocytic receptors that mediate the clearance of apoptotic cells. This study aimed to identify the role of MFG-E8 and TAM receptors in the development of DN.

Methods: A total of 146 patients with type 2 diabetes mellitus (T2DM), early stage DN, clinical DN and 48 healthy controls were employed to analyze the serum levels of MFG-E8, soluble Tyro3, Axl, Mer, and RAGE by enzyme-linked immunosorbent assay. The serum levels of CREA, hsCRP, CysC, and β2-microglobulin were measured by spectrophotometric analysis using a biochemical analyzer (AU5800).

Results: Our results showed that the serum levels of MFG-E8 were elevated in patients with T2DM compared with healthy controls; however, it decreased gradually in patients with DN with the severity of kidney injury, especially in the clinical DN group. Moreover, the levels of sTyro3, sAxl, and sMer were reduced in patients with T2DM and DN compared to healthy controls, particularly in patients with DN. The levels of MFG-E8, sTyro3, sAxl, and sMer were negatively correlated with UAER at 24 hours, CREA, hsCRP, CysC, β2-microglobulin, and RAGE, respectively. In addition, TAM receptors had significantly higher predictive and diagnostic values for early stage DN from T2DM than hsCRP, β2-microglobulin, and CysC, which are also predictive biomarkers of early stage DN from clinical DN.

Conclusions: Decreased MFG-E8 and TAM receptor expression is associated with an increased risk of microvascular complications in patients with T2DM, which plays a critical role in the diagnosis of diabetic patients with microvascular complications, especially early stage DN, and in monitoring the development of DN.

Abbreviations: DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus, UAER = urine albumin excretion rate.

Keywords: Axl, diabetic nephropathy, Mer, MFG-E8, Tyro3

1. Introduction

The incidence of diabetes mellitus is rising with the contribution of hyperglycemia, hypertension, obesity, hereditary diseases, smoking, advancing age, etc. Diabetes mellitus induces a persistent hyperglycemic state, which may lead to severe microvascular and macrovascular complications.[1] Diabetic nephropathy (DN), a major microvascular complication of diabetes mellitus, is the primary cause of end-stage renal disease. DN is characterized by the development of proteinuria and a reduction in the glomerular filtration rate, whereas the symptoms are not obvious in early stage DN.[1,2] Increasing evidence suggests that hyperglycemia, oxidative stress, and diabetic complications are closely related. An imbalance in oxidant-antioxidant homeostasis results in oxidative stress, leading to cellular damage in pathological conditions such as T2DM.[3] Inhibition of oxidative stress ameliorates the manifestations of DN, and overexpression of superoxide dismutase in streptozotocin-induced DN alleviates diabetic renal injury.[4] Apoptosis has been

YX and BW contributed equally to this work.

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Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

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discovered in renal tubular epithelial cells, endothelial cells, and stromal cells in DN. Persistent oxidative stress in a state of hyperglycemia can damage mitochondrial genetic material and nuclear DNA, which may accelerate ROS generation and induce cell necrosis or apoptosis.[5]

Milk fat globule-epidermal growth factor 8 (MFG-E8), a lipophilic glycoprotein secreted by mammary epithelial cells, monocytes, dendritic cells, and macrophages, acts as a bridging molecule between apoptotic cells and phagocytic cells by recognizing phosphatidylserine on the surface of apoptotic cells to mediate the clearance of apoptotic cells.[6] Several studies have reported that MFG-E8 participates in the development of diabetes mellitus and microvascular complications including diabetic peripheral neuropathy and diabetic retinopathy, and decreased MFG-E8 expression may increase the risk of developing diabetic peripheral neuropathy and diabetic retinopathy.[7,8]

The TAM receptor tyrosine kinases Tyro3, Axl, and Mer and the ligands Gas6/Protein S play a critical role in the phagocytosis of apoptotic cells to maintain homeostasis.[9] The loss of Tyro3 and protein S is associated with accelerated renal podocyte injury in diabetic mice.[10] Therefore, the aim of the present study was to identify the diagnostic value of MFG-E8 and TAM receptors in the development of DN in differentiating different stages of microvascular complications of T2DM.

2. Materials and methods

2.1. Patients

A total of 194 subjects (146 T2DM patients and 48 healthy controls) from Wuhan No.1 Hospital were enrolled between January 2018 and January 2019. The diagnosis of T2DM was based on the Guidelines for Prevention and Treatment of Type 2 Diabetes in China (2017 edition), and subjects with underlying diseases such as infection, tumor, liver cirrhosis, acute and chronic nephritis, and congestive heart failure were excluded. According to urinary albumin excretion rate (UAER) in 24 hours, the diabetes mellitus patients were classified into simple T2DM group (UAER < 30mg/24h), early-stage DN group (UAER: 30~300 mg/24h), and clinical DN group (UAER > 300mg/24h). Blood samples were collected, and sera were isolated by centrifugation and stored at -80 degrees centigrade until analysis by enzyme-linked immunosorbent assay. EDTA-anticoagulant blood was collected for the detection of glycosylated hemoglobin (HbA_{1C}) (%). Urine was collected at 24 hours and examined immediately. Written informed consent was obtained from all the study subjects. The study protocol was approved by the Ethics Committee of Wuhan No.1 Hospital.

2.2. Measurement of biochemical indicators

Serum levels of glucose (mmol/L), β2-microglobulin (mg/L), creatinine (CREA) (μmol/L), CysC (mg/L), and hsCRP (mg/L) were measured using an AU5800 automatic biochemical analyzer (Beckman Coulter, USA). Urine albumin at 24 hours was detected by immunoturbidimetry using a BNII automatic protein analyzer (Siemens, Germany). HbA_{1C} in the blood was detected by high-performance liquid chromatography using a D10 HBA1c analyzer (Bio-Rad).

2.3. Enzyme-linked immuno-sorbent assay (ELISA)

The double antibody sandwich enzyme-linked immunosorbent assay was used to detect the serum concentrations of MFG-E8

Table 1

Characteristics of the study subjects.

Characteristics	HC	T2DM	early stage DN	clinical DN
Number	48	50	57	39
Age (yr)	58 (42–76)	61 (35–76)	63 (37–90)	59 (35–80)
Gender (n, male)	20	24	27	19
Fasting glucose (mmol/L)	4.3 (0.45)	6.94 (2.60)*	7.62 (2.99)*	7.34 (4.32)*
HbA1C (%)	5.3 (0.52)	7.5 (2.43)*	8.9 (3.62)*,**	9.7 (3.26)*,**
UAER (mg/24h)	-	7.69 (11.05)	78.5 (53.65)**	486 (502.5)**,***
CREA (μmol/L)	57.8 ± 10.5	58.2 ± 13.1	73.2 ± 24.3	86.8 ± 49.6*,**
CysC (mg/L)	0.67 ± 0.16	0.93 ± 0.21	1.09 ± 0.31*	1.42 ± 0.65*,**,***
β2-microglobulin (mg/L)	1.32 ± 0.41	1.59 ± 0.48	1.94 ± 0.57*	2.16 ± 1.05*,**
hsCRP (mg/L)	0.18 ± 0.11	0.43 ± 0.75	1.22 ± 1.68	3.48 ± 3.62*,**,***

Data are described as median (range), median (interquartile range) or mean ± SD unless otherwise stated. HC, healthy control; T2DM, type 2 diabetes mellitus (UAER < 30 mg/24h); early-stage DN, T2DM patients with early stage diabetic nephropathy (UAER: 30–300 mg/24h); clinical DN, T2DM patients with clinical diabetic nephropathy (UAER > 300 mg/24h). CREA = creatinine, CysC = cystatin C, DN = diabetic nephropathy, hsCRP = hypersensitive C-reactive protein, T2DM = type 2 diabetes mellitus, UAER = urine albumin excretion rate.

*P < .05 for all T2DM patients compared with healthy controls.

**P < .05 for patients with early stage DN or clinical DN compared with T2DM patients.

***P < .05 for patients with clinical DN compared with early stage DN.

Table 2

Serum levels of MFG-E8, sTyro3, sAxl, sMer, and RAGE in patients with DM, early stage DN, clinical DN, and healthy individuals.

Biomarker	HC	T2DM	Early stage DN	Clinical DN
MFG-E8 (ng/mL)	2.09 ± 3.33	12.43 ± 12.33*	7.61 ± 12.73*,**	6.69 ± 9.58**
sAxl (ng/mL)	13.4 ± 10.58	8.38 ± 10.73*	4.2 ± 3.6*	5.36 ± 2.33*
sMer (ng/mL)	1.15 ± 0.44	1.4 ± 0.67	0.73 ± 0.55*,**	0.3 ± 0.28*,**,***
sTyro3 (ng/mL)	1.61 ± 0.4	1.41 ± 0.28*	1.16 ± 0.4*,**	0.76 ± 1.43*,**,***
RAGE (ng/mL)	0.066 ± 0.033	0.073 ± 0.05	0.118 ± 0.053*,**	0.188 ± 0.126*,**,***

Data are present as mean ± SD unless otherwise stated. HC, healthy control; T2DM, type 2 diabetes mellitus (UAER < 30 mg/24h); early stage DN, T2DM patients with early stage diabetic nephropathy (UAER: 30–300 mg/24h); clinical DN, T2DM patients with clinical diabetic nephropathy (UAER > 300 mg/24h). DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus, UAER = urine albumin excretion rate.

*P < .05 for all patients compared with healthy controls.

**P < .05 for patients with early stage DN or clinical DN compared with T2DM patients.

***P < .05 for patients with clinical DN compared with early stage DN.

(ng/mL) (Sigma), sTyro3 (ng/ml) (diluted 1:5, R&D Systems), sAxl (ng/mL) (diluted 1:10, R&D Systems), sMer (ng/mL) ((R&D Systems), and RAGE (ng/mL) (R&D Systems). The Capture antibodies were diluted with 1 × PBS and incubated at 4 °C overnight. After washing 3 times with 0.05% PBST, the boards were blocked with 5% bovine serum albumin for 2 hours. Then, diluted standard substances and serum specimens were added to the boards, incubated for 2 hours at room temperature, and washed again. Next, the detection antibodies were added and incubated for 2 hours at room temperature. After washing, streptavidin conjugated to horseradish peroxidase was added, followed by incubation for 20 minutes. The stopping solution was then added and the optical density (OD) at 450 nm was determined. Finally, the concentrations of MFG-E8, sTyro3, sAxl, sMer, and RAGE were calculated according to the standard curves.

2.4. Statistical analysis

All statistical analyses were performed using SPSS software 16.0. The clinical quantitative data were described as mean ± standard deviation (x ± s) in accordance with a normal distribution, and data that were not consistent with a normal distribution were described as median ± interquartile range. Comparisons between 2 independent groups were analyzed using an unpaired Student *t* test in accordance with a normal distribution. More than 2 groups were compared using one-way analysis of variance. The Mann-Whitney *U*-test was used to analyze 2 groups that were not consistent with a normal distribution, and more than 2 groups were compared using the Kruskal-Wallis *H*-test. The correlation between MFG-E8, sTyro3, sAxl, sMer, RAGE, hsCRP, CysC, CREA, β2-microglobulin, and UAER was calculated by pairwise Spearman analysis, and correlation strength with an absolute correlation $|r|>0.2$ was selected. The selected connections were plotted as an undirected network graph, with the width of the edge proportional to the correlation strength. The diagnostic values of selected parameters were assessed by ROC analysis and the area under ROC curve (AUC). Differences between groups were considered statistically significant if the *P* value was <.05.

3. Results

3.1. Cohort characteristics

The characteristics of the 194 patients are presented in Table 1. The 194 subjects were divided into 4 groups:48 healthy controls, 50 patients diagnosed with T2DM, 57 T2DM patients with early stage DN, and 39 T2DM patients with clinical DN. The age and sex of all patients were similar to those of the healthy controls. Patients with DN showed a significant increase in fasting glucose, HbA_{1C} and UAER compared to healthy subjects and T2DM patients ($P < .05$).

3.2. Elevated serum levels of CREA, CysC, β2-microglobulin and hsCRP in patients with DN

The serum CREA level was significantly higher in patients with clinical DN than in healthy controls or T2DM patients ($P < .05$, Table 1), but there was no significant difference between patients with early stage DN and clinical DN. The levels of CysC, β2-microglobulin, and hsCRP were elevated gradually in patients with declining renal function, particularly in clinical DN patients,

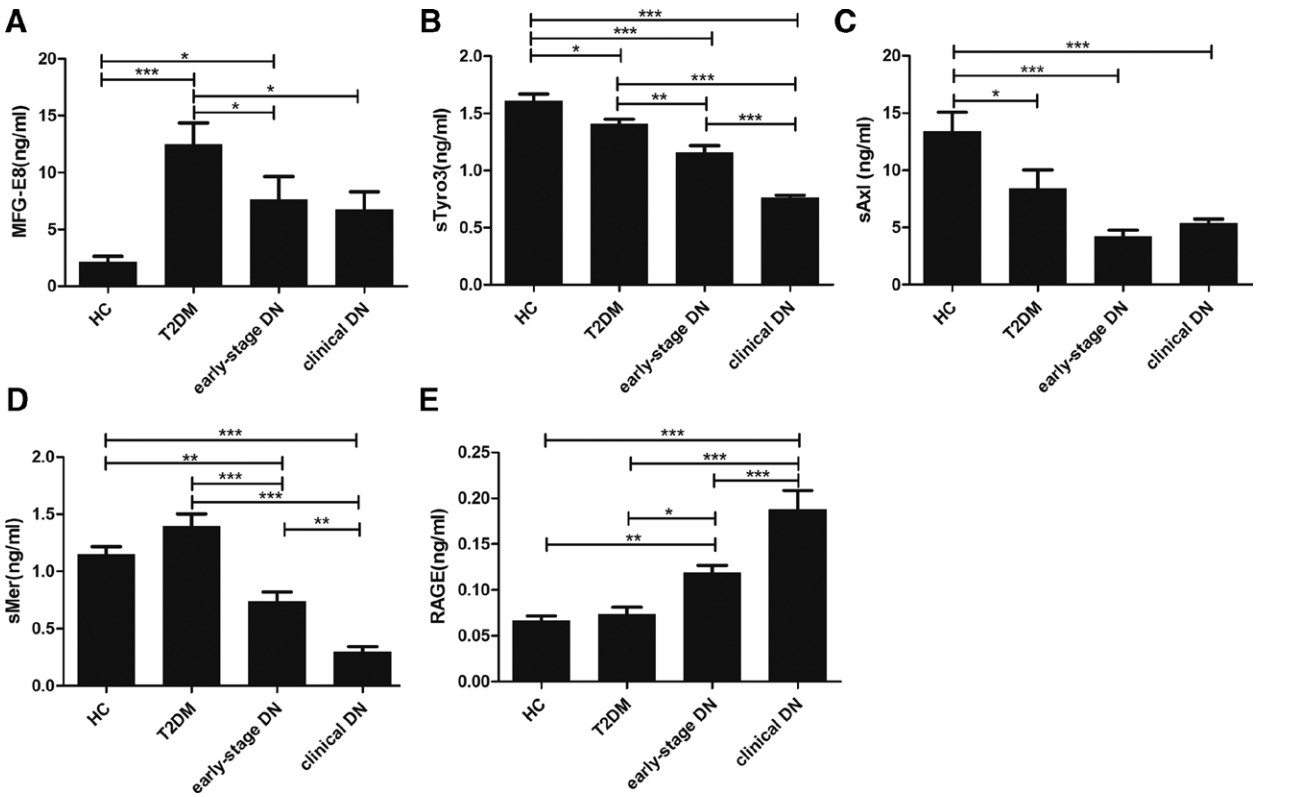

Figure 1. The serum levels of biomarkers in healthy controls and patients with T2DM and DN. The serum levels of MFG-E8 (A), sTyro3 (B), sAxl (C), sMer (D), and RAGE (E) was detected with ELISA. HC, healthy control (n = 48); T2DM, type 2 diabetes mellitus (UAER < 30 mg/24h, n = 50); early-stage DN, T2DM patients with early-stage diabetic nephropathy (UAER: 30–300 mg/24h, n = 57); clinical DN, T2DM patients with clinical diabetic nephropathy (UAER > 300 mg/24h, n = 39). Data represent as x ± s, statistical differences among groups were performed using one-way ANOVA, $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$. DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus, UAER = urine albumin excretion rate.

when compared to T2DM patients or healthy subjects ($P < .05$, Table 1). Notably, hsCRP levels were significantly enhanced in patients with clinical DN among these groups. These results demonstrate that increased levels of inflammatory mediators and damage-associated molecules are associated with the severity and development of DN in diabetic patients.

3.3. Decreased MFG-E8, sTyro3, sAxl, sMer and elevated RAGE expression were associated with diabetic patients with microvascular complications

Serum levels of MFG-E8, sTyro3, sAxl, sMer, and RAGE are shown in Table 2 and Figure 1. Serum levels of MFG-E8 were higher in patients with T2DM, early stage DN, and clinical DN than in healthy controls, particularly in patients with T2DM ($P < .05$). However, it decreased gradually in T2DM patients with microvascular complications (Fig. 1A). Compared to healthy individuals, the levels of soluble Tyro3 and Axl were decreased in patients with T2DM, early stage DN, and clinical DN, particularly in patients with clinical DN (Fig. 1B–C). In addition, the levels of sMer were significantly lower in patients with early stage and clinical DN than in diabetic patients and healthy individuals ($P < .05$), which showed no significant difference between T2DM patients and healthy subjects (Fig. 1D). In contrast, RAGE levels were remarkably elevated in patients with early stage and clinical DN compared with healthy controls or T2DM patients ($P < .05$, Fig. 1E). These results indicate that decreased circulating MFG-E8, sTyro3, sAxl, sMer, and increased RAGE expression might contribute to the development of DN in diabetic patients.

3.4. The correlation analysis of serum biomarkers in patients with DM and DN

Correlation analysis showed that the UAER level in patients with T2DM and DN was positively correlated with hsCRP ($R = 0.2712$), CysC ($R = 0.3182$), and CREA ($R = 0.3269$) (Fig. 2). Among the 10 circulating biomarkers in all patients, the levels of MFG-E8 was negatively correlated with UAER ($r = -0.1895$) or sAxl ($r = -0.1933$) (Fig. 2). Consistently, the levels of sTyro3 was negatively correlated with UAER ($r = -0.6912$), RAGE ($r = -0.3779$), CREA ($r = -0.2398$) or hsCRP ($r = -0.3241$). Moreover, sAxl levels were negatively correlated with RAGE ($r = -0.2623$) in patients with T2DM and DN, whereas it was positively correlated with UAER ($R = 0.1933$). In addition, a similar correlation was observed between sMer and UAER, CREA, hsCRP, β2-microglobulin, and RAGE ($r = -0.462$, $r = -0.2091$, $r = -0.2928$, $r = -0.2797$, $r = -0.3533$). In contrast, RAGE levels in patients with T2DM and DN were positively correlated with UAER, CREA, CysC, and β2-microglobulin ($R = 0.4065$, $R = 0.3791$, $R = 0.3896$, $R = 0.3148$). Therefore, decreased MFG-E8, soluble TAM receptors Tyro3, Axl, and Mer, and elevated RAGE were associated with renal inflammation and injury.

3.5. Potential serum biomarkers of predictive and diagnostic value for early stage DN and clinical DN from diabetic patients

ROC curves were drawn for patients with T2DM, early stage DN, clinical DN, and healthy individuals to evaluate the

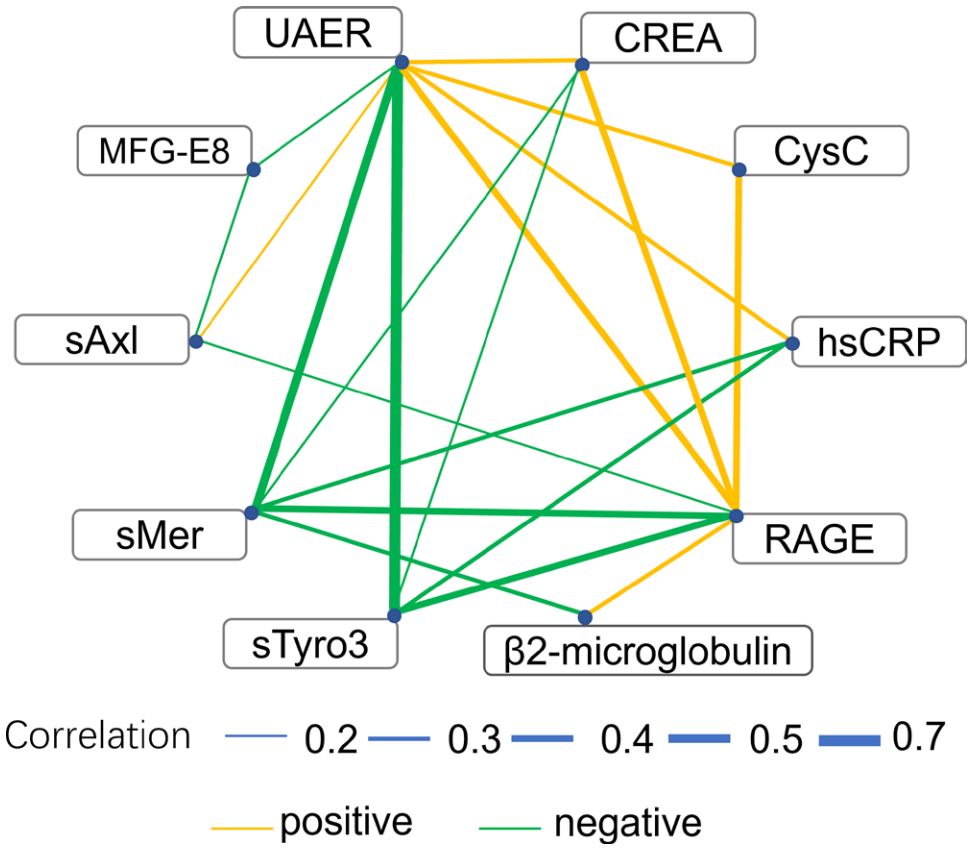

Figure 2. The correlation analysis of serum MFG-E8, sTyro3, sAxl, sMer, RAGE, hsCRP, CysC, CREA, β2-microglobulin and UAER in patients of T2DM (n = 50) and DN (n = 96) were analyzed using pairwise Spearman correlation analysis. Orange line represents positive relation, and green line represents negative relation. The width of the edge showing stronger or weaker interactions is proportional to the absolute value of biomarker-biomarker correlation (|*r*|). Edges were showed only when |*r*|>0.2. The correlation showed in this figure all has statistical difference, $P < .05$. DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus, UAER = urine albumin excretion rate.

predictive and diagnostic value of biomarkers for early-and clinical DN (Table 3) (Fig. 3). Although the AUC of sTyro3, sAxl, or sMer was <0.5, the AUC of the combined sTyro3, sAxl, and sMer was the largest among all biomarkers (AUC = 0.963, [95% CI] = 0.926–0.999), $P < 0.001$), suggesting that the combination of soluble TAM receptors is a potential biomarker for differentiating DN patients from healthy individuals. Furthermore, univariate logistic regression analysis showed that MFG-E8 (AUC = 0.658, [95% CI] = 0.533–0.784, $P = .023$), hsCRP (AUC = 0.885, [95% CI] = 0.810–0.960, $P < .001$), and the combination of β2-microglobulin and CysC (AUC = 0.873, [95% CI] = 0.793–0.953, $P < .001$) were also potential biomarkers for differentiating DN patients from healthy individuals (Fig. 3A).

To explore serum biomarkers to predict DN in patients with T2DM, we found that the combination of sTyro3, sAxl, and sMer (AUC = 0.967, [95% CI] = 0.931–1.004, $P < .001$), hsCRP (AUC = 0.848, [95% CI] = 0.742–0.954, $P < .001$), and β2-microglobulin and CysC (AUC = 0.842, [95% CI] = 0.741–0.943, $P < .001$) were potential biomarkers for predicting the development of DN from T2DM (Fig. 3B).

An analysis was performed to determine the importance of the development of early stage DN in T2DM (Fig. 3C). The analysis revealed that the combination of sTyro3, sAxl, and sMer (AUC = 0.851, [95% CI] = 0.740–0.961, $P < .001$), hsCRP (AUC = 0.748, [95% CI] = 0.647–0.920, $P = .001$), and the combination of β2-microglobulin and CysC (AUC = 0.767, [95% CI] = 0.623–0.910, $P = .002$) were predictive biomarkers of early stage DN in T2DM patients.

Further univariate logistic regression analysis showed that the combination of sTyro3, sAxl, and sMer (AUC = 0.803, [95% CI] = 0.627–0.935, $P = .001$), hsCRP (AUC = 0.977, [95% CI] = 0.944–1.011, $P < .001$), and the combination of β2-microglobulin and CysC (AUC = 0.791, [95% CI] = 0.655–0.927, $P = .001$) were potential biomarkers for predicting clinical DN in T2DM patients (Fig. 3D).

In addition, ROC curves of biomarkers from early stage and clinical DN patients were drawn (Fig. 3E). Analysis showed that sTyro3 (AUC = 0.855, [95% CI] = 0.741–0.969, $P < .001$), sMer (AUC = 0.838, [95% CI] = 0.719–0.951, $P < .001$), the combination of sTyro3, sAxl, and sMer (AUC = 0.836, [CI] = 0.713–0.959, $P < .001$), and the combination of β2-microglobulin and CysC (AUC = 0.844, [95% CI] = 0.710–0.979, $P < .001$) were significant predictors of the severity of microvascular complications in diabetes. Thus, our results demonstrated that the combination of soluble TAM receptors was significant for the early prediction of DN severity and progression in patients with diabetes.

4. Discussion

Inability to control glucose levels leads to major microvascular and macrovascular complications in diabetes. Among the various complications of diabetes, DN is observed in approximately 40% of patients, leading to end-stage renal disease and even death.[11] Immune system and inflammatory processes are involved in the occurrence and development of DN.[12] In this study, decreased MFG-E8 and TAM receptor expression were associated with an increased risk of microvascular complications in patients with T2DM, with its potential diagnostic value in monitoring the development of DN.

Hyperglycemia, derived from diabetes mellitus, can cause an increase in the formation of intracellular advanced glycation end products (RAGE). Our results showed a consistent increase in RAGE expression in diabetic patients with severe renal injury in DN. RAGE binds to receptors on podocytes and mesangial or tubular epithelial cells and induces the generation of reactive oxygen species, which activate transcription factors and release inflammatory cytokines via mitochondrial electron transport chain response and glucose oxidation.[3,13] Increasing studies have shown that oxidative stress generates adverse synergic effects by metabolic attacks on the molecules of target renal tissues and alters renal hemodynamics, resulting in endothelial dysfunction, inflammation, apoptosis, and necrosis, which are closely related to the impairment of renal function.[14] Apoptosis played a critical role in homeostasis and is induced by the activation of intracellular signaling pathways such as phosphoinositide 3-kinase (PI3K)/Akt.[6] Emerging evidence has demonstrated that podocyte apoptosis is regulated by Bcl2/Bax under hyperglycemic conditions in DN.[15]

MFG-E8 is a multifunctional glycoprotein that exerts a regulatory role in intercellular interactions involved in a variety of biological and pathological processes, including inflammatory diseases and diabetic vascular complications.[16] Compared with healthy subjects, the serum level of MFG-E8 was significantly higher in T2DM and DN patients, which was in line with previous studies showing that the expression of MFG-E8 was increased in T2DM patients and in diabetic kidneys of db/db

Table 3

Potential biomarkers of predictive and diagnostic value for early-stage DN and clinical DN.

Biomarker	AUC	95% CI	*P* value
HC *vs* DN			
MFG-E8	0.658	0.533–0.784	.023
sAxl	0.345	0.206–0.484	.026
sMer	0.179	0.082–0.275	<.001
sTyro3	0.081	0.018–0.144	<.001
hsCRP	0.885	0.810–0.960	<.001
β2-microglobulin + CysC	0.873	0.793–0.953	<.001
sTyro3 + sAxl + sMer	0.963	0.926–0.999	<.001
T2DM *vs* DN			
MFG-E8	0.370	0.220–0.521	.100
sAxl	0.474	0.294–0.655	.745
sMer	0.176	0.075–0.276	<.001
sTyro3	0.061	0.003–0.119	<.001
hsCRP	0.848	0.742–0.954	<.001
β2-microglobulin + CysC	0.842	0.741–0.943	<.001
sTyro3 + sAxl + sMer	0.967	0.931–1.004	<.001
T2DM *vs* early-stage DN			
MFG-E8	0.363	0.196–0.530	.111
sAxl	0.378	0.214–0.542	.156
sMer	0.208	0.077–0.339	.001
sTyro3	0.209	0.132–0.451	.015
hsCRP	0.784	0.647–0.920	.001
β2-microglobulin + CysC	0.767	0.623–0.910	.002
sTyro3 + sAxl + sMer	0.851	0.740–0.961	<.001
T2DM *vs* clinical DN			
MFG-E8	0.304	0.147–0.462	.026
sAxl	0.536	0.348–0.725	.681
sMer	0.014	0.011–0.040	<.001
sTyro3	0.023	0.012–0.057	<.001
hsCRP	0.977	0.944–1.011	<.001
β2-microglobulin + CysC	0.791	0.655–0.927	.001
sTyro3 + sAxl + sMer	0.803	0.627–0.935	.001
early-stage DN *vs* clinical DN			
MFG-E8	0.558	0.381–0.735	.528
sAxl	0.341	0.170–0.511	.083
sMer	0.838	0.719–0.958	<.001
sTyro3	0.855	0.741–0.969	<.001
hsCRP	0.272	0.117–0.427	.013
β2-microglobulin + CysC	0.844	0.710–0.979	<.001
sTyro3 + sAxl + sMer	0.836	0.713–0.959	<.001

Univariate logistic regression analysis of MFG-E8, sTyro3, sMer, sAxl, hsCRP, the combination of β2-microglobulin and CysC (β2-microglobulin + CysC), and the combination of sTyro3, sAxl and sMer (sTyro3 + sAxl + sMer) in patients with T2DM, early stage DN and clinical DN is conducted by SPSS software (16.0). The AUC, 95% CI and *P* value are analyzed and calculated.

DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus.

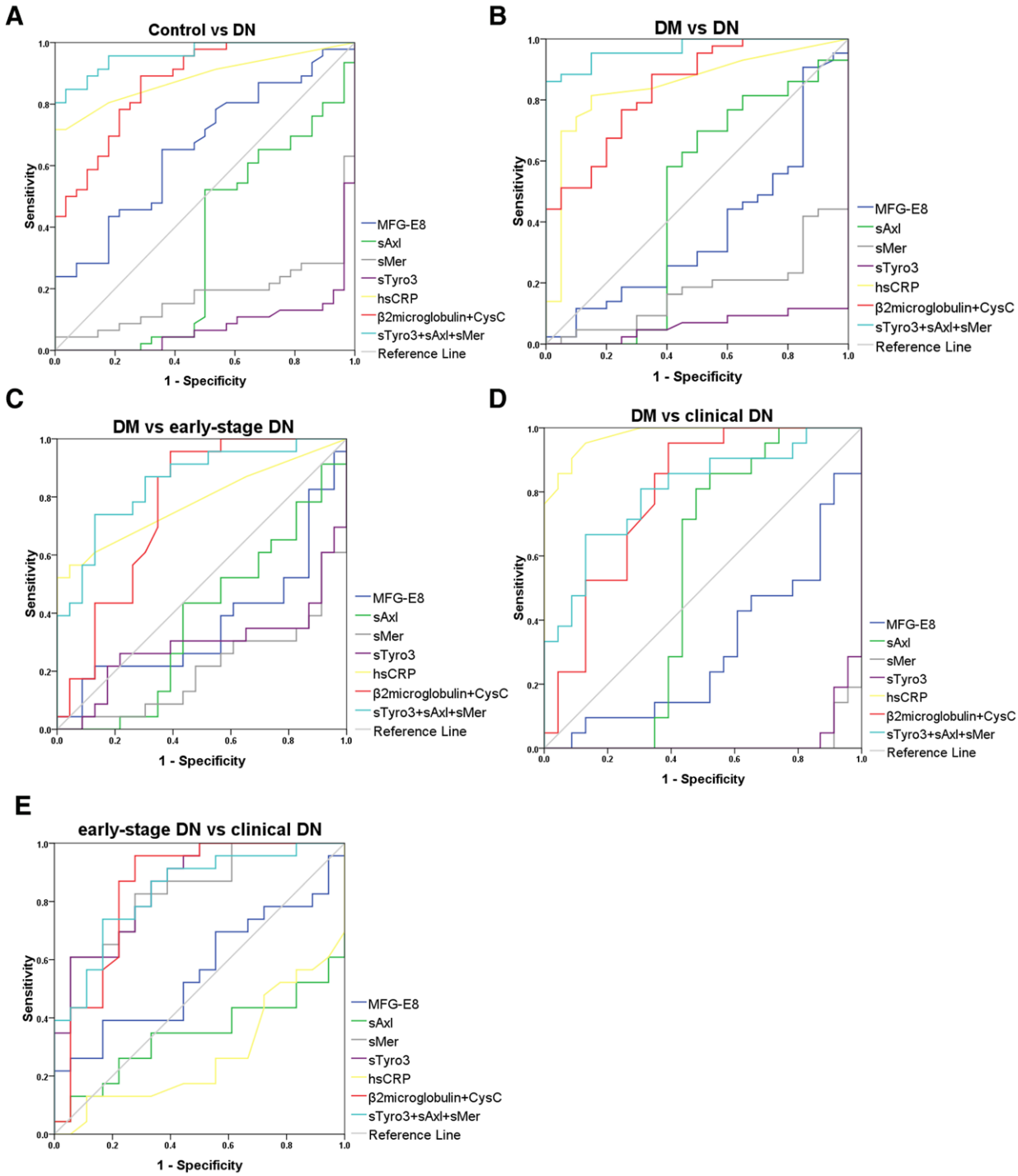

Figure 3. Univariate logistic regression analysis and ROC curves of serum biomarkers in patients with T2DM, DN, and healthy individuals. (A) The ROC curves of MFG-E8, sAxl, sMer, sTyro3, hsCRP, the combination of β2-microglobulin and CysC (β2-microglobulin + CysC), the combination of sTyro3, sAxl and sMer (sTyro3 + sAxl + sMer) in healthy controls and patients with DN. (B) The ROC curves of MFG-E8, sAxl, sMer, sTyro3, hsCRP, β2-microglobulin + CysC, and sTyro3 + sAxl + sMer in patients with T2DM and DN. (C) The ROC curves of MFG-E8, sAxl, sMer, sTyro3, hsCRP, β2-microglobulin + CysC, sTyro3 + sAxl + sMer in patients with T2DM and early-stage DN. (D) The ROC curves of MFG-E8, sAxl, sMer, sTyro3, hsCRP, β2-microglobulin + CysC, sTyro3 + sAxl + sMer in patients with T2DM and clinical DN. (E) The ROC curves of MFG-E8, sAxl, sMer, sTyro3, hsCRP, β2-microglobulin + CysC, sTyro3 + sAxl + sMer in patients with early-stage DN and clinical DN. DN = diabetic nephropathy, MFG-E8 = milk fat globule-epidermal growth factor 8, T2DM = type 2 diabetes mellitus.

mice.[16,17] Increased MFG-E8 along with activation of ERK1/2, Akt, and GSK-3β signaling pathways contributes to the pathogenesis of DN, accounting for a variety of biological events, including mesangial cell-cycle progression, hypertrophy, and extracellular matrix protein synthesis.[17] Lin et al confirmed that high serum MFG-E8 expression is positively correlated with aortic stiffness and renal function impairment.[18] However, the expression of MFG-E8 in patients with early stage and clinical DN was significantly lower than that in T2DM patients. Sun et al consistently reported that a lower serum MFG-E8 concentration was significantly associated with an increased risk of microvascular complications in T2DM patients.[7] The

lessened expression of serum MFG-E8 may reflect its increased consumption during the removal of large numbers of apoptotic proximal tubule epithelial, endothelial, and interstitial cells in renal tissues in DN.[19,20] Correction of MFG-E8 can resolve inflammation and promote cutaneous wound healing, accompanied by elimination of apoptotic cells in the wound.[21] Our previous study also showed that as inhibitor of the "NLRP3 inflammasome-NETs" inflammatory loop, exogenous recombinant MFG-E8 improves angiogenesis and accelerates wound healing in diabetes.[22]

TAM receptor tyrosine kinases Tyro3, Axl, and Mer contribute to cell survival, proliferation, migration, and clearance of apoptotic cells.[23,24] In this study, the serum levels of soluble TAM receptors Tyro3, Axl, and Mer were significantly lower in patients with DN than in diabetic patients or healthy individuals, and decreased gradually with the severity of renal microvascular impairment. The serum levels of sTyro3 and sMer were negatively correlated with UAER and CREA, suggesting that the limitation of soluble TAM receptors is associated with the development of kidney injury. Tyro3 mRNA expression is highly enriched in human glomeruli and Tyro3 protein is expressed in podocytes. Glomerular Tyro3 mRNA expression increased in mild DN but was suppressed in progressed DN.[25] The decreased Tyro3 expression due to activation of the TNF-α/NF-κB pathway in progressive diabetic kidney disease.[25] Zhong et al found that protein S-induced activation of Tyro3 receptors conferred protection against podocyte injury, and the loss of the protein S/Tyro3 signaling pathway aggravated diabetic kidney injury.[10] Recent study showed that Gas6, Axl, and soluble Axl concentrations were significantly lower in diabetic patients and were negatively correlated with eGFR, suggesting a potential predictive value for DN.[26] Upregulation of Gas6/Axl signaling is a protective mechanism that reduces tubule-interstitial apoptosis and slows the progression to end-stage renal failure in DN.[27] However, Peter *et al* showed that patients with macroalbuminuria diabetes had higher circulating levels of sMer, and increased sTyro3 and sMer levels were associated with loss of tubular Tyro3 and Mer expression in DN tissue and glomerular deposition of protein S.[28] We speculated that the steadily decreasing expression of soluble TAM receptor could be attributed to aggravating podocytes, endothelial cells, and other glomerular cell injury in early stage and clinical DN patients in our study. Moreover, circulating levels of sMer and sTyro3 were negatively correlated with UAER, CREA, hsCRP, and β2 microglobulin. Nevertheless, our study demonstrated that decreased TAM receptors were associated with the severity of kidney injury in diabetes, exhibiting a protective role in progressive DN.

Because of the insidious onset of DN, preventive treatment is difficult when the clinical manifestations are proteinuria, edema, and renal insufficiency. However, DN can be reversed after early clinical intervention; thus, early diagnosis of renal injury is of great significance in diabetes. Emerging evidence has shown that serum CysC, hsCRP, and β2-microglobulin levels are sensitive markers of early diabetic renal damage.[29] In our study, hsCRP and combined detection of β2-microglobulin and CysC showed high sensitivity and specificity for the diagnosis of early DN or clinical nephropathy from diabetes. Furthermore, the detection of MFG-E8, sTyro3, or sMer is significant in the differential diagnosis of early stage DN and clinical DN. Interestingly, the combined detection of sTyro3, sAxl, and sMer had significantly higher predictive and diagnostic values for early stage DN from T2DM than hsCRP or combined detection of β2-microglobulin and CysC. The combination of sTyro3, sAxl, and sMer detection also had a greater significance in the differential diagnosis of early stage DN and clinical DN, as well as sTyro3 or sMer. Our results suggest that the TAM receptors sTyro3, sAxl, and sMer can serve as biomarkers to predict and monitor the development and progression of early stage DN and distinguish DN from T2DM, which is of great significance for the clinical prevention and treatment of early DN and monitoring the progression to end-stage renal disease.

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References

[1] World Health Organization, "Global report on diabetes", April 2016, Global report on diabetes (who.int).
[2] International Diabetes Federation, "Atlas of diabetes," Seventh edition, 2015, December 2017, http://www.diabetesatlas.org.
[3] Jin Q, Tongtong L, Yuan Q, et al. Oxidative stress and inflammation in diabetic nephropathy: role of polyphenols. Front Immunol. 2023;14:1185317.
[4] Thallas-Bonke V, Thorpe SR, Coughlan MT, et al. Inhibition of NADPH oxidase prevents advanced glycation end product-mediated damage in diabetic nephropathy through a protein kinase C-alpha-dependent pathway. Diabetes. 2008;57:460–9.
[5] Sonia S-F, Enrique Padilla-Tejeda D, Sandra C-I, et al. Oxidative stress, apoptosis, and mitochondrial function in diabetic nephropathy. Int J Endocrinol. 2018;1:13.
[6] Xiao Y, Li G, Chen Y, et al. Milk fat globule-epidermal growth factor-8 pretreatment attenuates apoptosis and inflammationviathe integrin-β3 pathway after surgical brain injury in rats. Front Neurol. 2018;9:96.
[7] Guohua S, Juxiang L, Guanghao X, et al. Reduced serum milk fat globule-epidermal growth factor 8 (MFG-E8) concentrations are associated with an increased risk of microvascular complications in patients with type 2 diabetes. Clin Chim Acta. 2017;466:201–6.
[8] Yasmen Sabry M, Mohamed Watany M, Abbas Ghali A, et al. Study of Serum Milk Fat Globule-epidermal Growth Factor 8 (MFG-E8) in patients with type 2 diabetes mellitus. J Adv Med Med Res. 2021;1:11.
[9] Aehnlich P, Powell RM, Peeters MJW, et al. TAM receptor inhibition-implications for cancer and the immune system. Cancers (Basel). 2021;13:1195.
[10] Fang Z, Haibing C, Yifan X, et al. Protein S protects against podocyte injury in diabetic nephropathy. J Am Soc Nephrol. 2018;29:1397–410.
[11] Namrata A, Pritam S, Sukanya S, et al. Therapeutic insights against oxidative stress induced diabetic nephropathy: a review. J Autoimmune Dis. 2015;1:1–16.
[12] Chen J, Liu Q, He J, et al. Immune responses in diabetic nephropathy: Pathogenic mechanisms and therapeutic target. Front Immunol. 2022;13:958790.
[13] Wu XQ, Zhang DD, Wang YN, et al. AGE/RAGE in diabetic kidney disease and ageing kidney. Free Radic Biol Med. 2021;171:260–71.
[14] Nam SM, Lee MY, Koh JH, et al. Effects of NADPH oxidase inhibitor on diabetic nephropathy in OLETF rats: the role of reducing oxidative stress in its protective property. Diabetes Res Clin Pract. 2009;83:176–82.
[15] YaMin F, Sheng C, JiaRong X, et al. Dysregulation of lncRNAs GM5524 and GM15645 involved in high glucose induced podocyte apoptosis and autophagy in diabetic nephropathy. Mol Med Rep. 2018;18:3657–64.
[16] Fei Y, Bao-ying Li, Xiao-li Li, et al. Proteomic analysis of aorta and protective effects of grape seed procyanidin B2 in db/db mice reveal a critical role of milk fat globule epidermal growth factor-8 in diabetic arterial damage. PLoS One. 2012;7:e5241.
[17] Zhen Z, Bao-ying Li, Xiao-li Li, et al. Proteomic analysis of kidney and protective effects of grape seed procyanidin B2 in db/db mice indicate

MFG-E8 as a key molecule in the development of diabetic nephropathy. Biochim Biophys Acta. 2013;1832:805–16.
[18] Lin YP, Hsu ME, Chiou YY, et al. Comparative proteomic analysis of rat aorta in a subtotal nephrectomy model. Proteomics. 2010;10:2429–43.
[19] Allen DA, Harwood S, Varagunam M, et al. High glucose-induced oxidative stress causes apoptosis in proximal tubular epithelial cells and is mediated by multiple caspases. FASEB J. 2003;17:908–10.
[20] Droge W. Free radicals in the physiological control of cell function. Physiol Rev. 2002;82:47–95.
[21] Mitava D, Subhadip G, Mithun S, et al. Correction of MFG-E8 resolves inflammation and promotes cutaneous wound healing in diabetes. J Immunol. 2016;196:5089–100.
[22] Huang W, Jiao J, Liu J, et al. MFG-E8 accelerates wound healing in diabetes by regulating "NLRP3 inflammasome-neutrophil extracellular traps" axis. Cell Death Discov. 2020;6:84.
[23] Rothlin CV, Carrera-Silva EA, Bosurgi L, et al. TAM receptor signaling in immune homeostasis. Annu Rev Immunol. 2015;33:355–91.
[24] Vago JP, Amaral FA, van de Loo FAJ. Resolving inflammation by TAM receptor activation. Pharmacol Ther. 2021;227:107893.
[25] Zhong F, Chen Z, Zhang L, et al. Tyro3 is a podocyte protective factor in glomerular disease. JCI Insight. 2018;3:e123482.
[26] Özel M, Başkol G, Abedi AH, et al. Evaluation of serum growth arrest specific-6/sAXL levels in type 2 diabetes mellitus. Erciyes Med J. 2022;44:1–14.
[27] Hyde GD, Taylor RF, Ashton N, et al. Axl tyrosine kinase protects against tubulo-interstitial apoptosis and progression of renal failure in a murine model of chronic kidney disease and hyperphosphataemia. PLoS One. 2014;9:e102096.
[28] Peter O, Lionel L, Mohamed A, et al. Increased circulating and urinary levels of soluble TAM receptors in diabetic nephropathy. Am J Pathol. 2017;187:1971–83.
[29] Chen H, Li H. Clinical implication of cystatin c and β2-microglobulin in early detection of diabetic nephropathy. Clin Lab. 2017;63:241–7.