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Original article

Midkine: As a Predictor of Early Diabetic Nephropathy in Children with Type 1 Diabetes Mellitus

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What is already known on this topic?
Microalbuminuria is the gold standard for the detection and prediction of DN. However , several studies indicating the non-specificity of microalbuminuria for accurate prediction of DN.

What this study adds?
Serum MK is a useful, novel, practical marker for the evaluation of renal involvement in children with T1DM, especially in normoalbuminuric children.

Abstract
Objectives: This study was aimed to assess the role of serum midkine (MK) as a biomarker for early detection of diabetic nephropathy **(**DN) in children with type 1 diabetes mellitus (T1DM) before microalbuminuria emerges.
Methods: A total of 120 children with T1DM, comprising 60 microalbuminuric patients (group 1), 60 normoalbuminuric patients (group 2), and 60 healthy participants as a control group (group 3) were included in this study. Detailed medical history, clinical examination, and laboratory assessment of high-sensitivity C-reactive protein (hs- CRP) , hemoglobin A1c% (HbA1c%) , lipid profile , urinary albumin creatinine ratio (ACR), serum MK, and estimated glomerular filtration rate based on serum creatinine (eGFR-Cr) were performed in the three studied group.
Results: Our study revealed significantly higher serum MK in both diabetic groups compared to controls ($p < 0.001$). Additionally, patients with microalbuminuria had higher serum MK concentrations than those with normoalbuminuria ($p<0.001$). The normoalbuminuric group had highly significantly elevated serum MK compared with the control group ($p < 0.001$). Receiver operating characteristic (ROC) curve analysis revealed that MK cutoff value of 1512 pg/ml was able to predict microalbuminuria with a sensitivity of 96% and specificity of 92%. Stepwise regression analysis revealed that HbA1c %, hs- CRP, and urinary ACR were independently related to MK levels ($p < 0.001$ for each).
Conclusions: The results of this study suggest that serum MK is a useful, novel, practical marker for the evaluation of renal involvement in children with T1DM, especially in normoalbuminuric children.
Keywords: Midkine, Type 1 Diabetes Mellitus, diabetic nephropathy, urinary albumin creatinine ratio

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Introduction

Midkine (MK) is a multifunctional heparin-binding growth factor that was primarily identified as the retinoic acid–response gene product [1]. It has pleiotropic activities as it enhances cell proliferation, differentiation, survival, migration and also involved in angiogenesis and oncogenesis [2,3]. Furthermore, functional evidence has confirmed its possible role in modifying inflammatory responses [4]. MK is pathologically implicated in multiple disease processes, including cancer development, neuronal survival , tissue inflammation, and acute and chronic kidney disease [5-7]. In the kidney, MK is expressed in both proximal tubular cells and distal tubular epithelial cells [7] and to a minor extent in endothelial cells [8] and is induced by oxidative stress via the activation of hypoxia-inducible factor-1-alpha, [7]. The pathological roles of MK in renal disease are broad, ranging from the chronic kidney disease (CKD) progression [8], hypertension [9] , diabetes mellitus (DM) [10] and drug toxicity [11].

Diabetic nephropathy [DN] is a grave complication caused by both type 1 and 2 DM and, unless arrested, leads to end-stage kidney disease [12]. Pathologically, it is a diffuse process affecting glomerular endothelial cells, tubular epithelial cells, and interstitium [13]. It progress through glomerular hyperfiltration, silent phase (normoalbuminuria), incipient nephropathy (microalbuminuria), overt nephropathy (macroalbuminuria), and established renal failure [14,15].The occurrence of the glomerular basement membrane and tubular basement membrane thickening on histopathology of T1DM suggests that tubular injury is not dependent of glomerular injury as both can occur after a same duration of disease [16]. As with other kidney diseases, the outcome of diabetic kidney disease (DKD) is better determined by tubulointerstitial changes than glomerular changes [17]. Classically, microalbuminuria is the gold standard for the detection and prediction of DN [12]. However, studies had shown that histopathologic changes of DN may occur in patients with normoalbuminuric diabetic patients[18]. Furthermore, microalbuminuria appears once significant renal damage has actually occurred[19].Moreover, microalbuminuria has several confounding factors associated with it such as urinary tract infection, exercise and acute illness indicating the non-specificity of microalbuminuria for accurate prediction of DN[20].Consequently, there is a urgent need for a more specific and sensitive biomarker for earlier discovery of DN during the “tubular stage” of renal damage before microalbuminuria appears .Up to our best knowledge, no data is available regarding the association between serum MK and DN in children with T1DM. The current study aimed to assess the diagnostic values of serum MK as a novel biomarker in the prediction of microalbuminuria thus allows for early recognition of DN in children with T1DM before microalbuminuria appears .

Patients and Methods

Patients

This is a case-control study included sixty children and adolescents with type 1DM having microalbuminuria (urinary albumin excretion 30–299 mg/g creatinine) (microalbuminuric group)(group1)and sixty children and adolescents with type 1 DM with normoalbuminuric (urinary albumin excretion <30 mg/g creatinine) (normoalbuminuric group) (group 2) [21]. Exclusion criteria were the presence of any clinical or laboratory evidence of chronic infection, immunosuppression , liver diseases ,heparin therapy, connective tissue disease, or other autoimmune disorders Patients on antiplatelet drugs, lipid-lowering medication, or antihypertensive therapy including angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) were also excluded as the protective role of both ACEIs and ARBs on glomerular and tubulointerstitial compartments have been proved in human studies [22]. Patients were recruited over a period of 2 years (April 2017 to March 2019) from the outpatient pediatric diabetes clinic of Children’s Hospital, Assiut University, Assiut, Egypt. This study also included sixty healthy children and adolescents who were recruited from the general population and matched for age, sex, pubertal stage, BMI SDS, and socioeconomic status (group 3). The study protocol was approved by the Ethics Committee of Faculty of Medicine, Assiut Children’s University Hospital, Assiut, Egypt (33/2017). Informed consent was obtained from each patient or control subject or their legal guardians before enrollment into the study.

Methods

The studied patients were subjected to detailed medical history and clinical through examination with special emphasis on the age of onset of diabetes, disease duration, and insulin dose. Anthropometric measurements including weight, height, and waist were obtained by a trained nurse according to standardized techniques . Body mass index (BMI) was calculated as weight divided by squared height (kg/m2) using reference data for Egyptian children and adolescents and was expressed as standard deviation scores (SDSs) [22]. Puberty was assessed using the standardized method of Tanner stages [23]. Systolic and diastolic blood pressure (SBP and DBP) was measured by standard technique. Standard deviation scores (SDSs) for mean BP were calculated according to the report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Children and Adolescents [24]. Hypertension was defined as mean systolic or mean diastolic BP > 1.645 SDS (1.645 SDS corresponds to the 95th percentile in a standard normal distribution).

Laboratory investigations

Fasting lipid profile and high sensitivity C-reactive protein (hs-CRP) were measured using Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany). Serum total cholesterol, high-density lipoprotein (HDL), and triglyceride concentrations were measured by standard enzymatic methods using Boehringer Mannheim GmbH (Germany) reagents with a fully automated analyzer. Calculation of LDL concentration was performed using Friedewald's equation [25]. Assessment of mean HbA1c % in the year preceding the study was performed using High-performance liquid chromatography (Variant Analyzer; Bio-Rad, Inc., Cairo, Egypt). Urinary albumin excretion (as an indicator of nephropathy) was measured in an early morning urine sample as an albumin-to-creatinine ratio by an immuno-nephelometric method on a prime photometer (BCP BioSed, Rome, Italy). Microalbuminuria is present if urinary albumin excretion in at least two out of three consecutive urine samples, 2 months apart was 30–299 mg/g creatinine and normoalbuminuric if urinary albumin excretion (<30 mg/g creatinine)[21]. Potential factors affecting urinary albumin excretion as exercise, fever, posture were excluded [26]. Serum creatinine (Cr, mg/dL) was measured on a Dimension Xpand plus chemistry analyzer using its kits; which were supplied by Siemens Technology (Illinois). Serum creatinine-based eGFR (eGFR-Cr) was calculated using the updated Schwartz formula. eGFR-Cr = 0.413*height (cm)/Serum Cr (mg/dL) [27]. Determination of serum MK by Human Midkine ELISA Kit provided by (Boster Biological Technology Company, Pleasanton, CA, USA).

Statistical analysis

Data were analyzed using IBM SPSS Statistics 19.0 (SPSS Inc., Chicago, IL, USA). Qualitative variables were presented as number and percent [n (%)] and compared by the Chi-square test. Quantitative variables were tested for normality using Kolmogrov-Smirnov test. Normally distributed quantitative variables were expressed as mean and SD (mean ± SD) and the one-way analysis of variance (ANOVA) test was used to compare the three studied groups with Bonferroni posthoc test used to detect pair-wise comparison. Spearman correlation was used for non-parametric correlation between quantitative variables and Pearson correlation was used for parametric correlation. Results were considered statistically significant for any test if $P < 0.05$. Multiple linear regression analysis was employed to assess the relationship between MK and clinical and laboratory variables Logistic regression was used to examine the relation between MK and ACR after adjustment of other variables Receiver operating characteristic (ROC) curve was used to determine the best cutoff value of MK in the prediction of microalbuminuria in children with T1DM . The area under the curve (AUC), specificity and sensitivity were computed based on the ROC. A p-value <0.05 was considered significant in all analyses.

Results

The demographic data and laboratory finding of the patient groups and the control group were shown in (Table 1) Children with type 1 diabetes in both groups had significantly higher SBP SDS, DBP SDS, serum total, and LDL cholesterol, triglycerides, HbA1c%, hs-CRP, ACR, and MK compared with control subjects ($p < 0.05$). Children with microalbuminuria were older with longer disease duration. They had significantly higher blood pressure SDS, HbA1c, hs-CRP, urinary ACR, serum lipids (except HDL cholesterol), and insulin dose compared with normoalbuminuric cases ($p < 0.05$). ROC curve analysis revealed that MK cutoff value of 1512 pg/ml was able to predict microalbuminuria with a sensitivity of 96% and specificity of 92% (area under the curve, 0.94; confidence interval (CI), 0.87–1.00 ;($p < 0.001$). (Fig 1).

MK was correlated positively with disease duration, systolic and diastolic blood pressure SDS, HbA1c, hs-CRP, and urinary ACR ($p < 0.05$) , while no correlation was reported between MK and age, serum lipids, BMI SDS, or insulin doses ($p > 0.05$) (Table 2) . Stepwise regression analysis (Table 3) revealed that HbA1c, hs-CRP, and urinary ACR were independently related to MK levels ($p = <0.001$). Moreover, logistic regression revealed that MK was a significant independent factor for DN after adjustment of other variables; age, gender, disease duration, BMI SDS, blood pressure, HbA1c, ACR, and fasting lipids (odds ratio 2.05, 95% CI 1.16–5.26; $p < 0.001$).

Discussion

In this study, MK levels were significantly higher in diabetic children with microalbuminuria compared with those with normoalbuminuria and controls. Most importantly, the normoalbuminuric children had significantly higher levels of MK compared with controls. This suggests that the serum MK levels are related to subclinical tubular impairment and can be used as earlier measurable marker of renal involvement before the onset of microalbuminuria .Furthermore, MK correlates positively and significantly with urinary ACR ($p<0.001$) suggesting that MK influence the severity of renal involvement and MK may be used as a marker to stratify DN into different stages. In a previous study, Kosugi T et al. [10]. reported that kidney biopsy tissue from eight adult patients with DN revealed that strong tubular atrophy, interstitial fibrosis and interstitial cell infiltration were evident in the specimens of DN, in which MK induction was detected in the glomeruli, tubules and interstitium, In addition, MK expression was detected in all the examined cases, which exhibited differently states of DN. These data were in agreement with the MK expression pattern in a mouse model induced by Streptozotocin. Although glomerular dysfunction is thought to be a major factor for the development and progression of DN, tubulointerstitial damage may also play an important role in the pathogenesis of DN. MK is up-regulated in damaged tubular epithelial cells during the extremely early phase in vivo and in vitro studies involving ischemia and hypoxia [28]. Experimental studies stated that

MK antisense oligodeoxynucleotides (anti-MK ODN) can improve ischemic reperfusion-induced renal damage , arterial restenosis and cisplatin-induced nephropathy [11]. In line with our results, we suggest that MK inhibitors may be useful in treating DN which may open up new avenues for the development of therapy for DN [29].

The International Society of Nephrology recommend annual screening for albuminuria and measurement of eGFR to detect and monitor DN in patients with DM . In the current study, no significant difference in eGFR-Cr among the studied groups was detected .Moreover, serum MK was not correlated with eGFR-Cr. These data indicating that serum MK is a better predictor of eGFR when compared to the classical eGFR-Cr method in children with T1DM, as eGFR-Cr was not able to reflect early renal affection in the present study.

In this study, HbA1c % was significantly higher in diabetic children with microalbuminuria (group 1) compared with children with normoalbuminuria (group2) and controls (group 3).Moreover, there was also a significant correlation between HbA1c and MK ($P<0.001$). Kosugi T et al. [10] reported that MK expression was up-regulated by high glucose in primary cultured tubular epithelial cells. They also identify MK as a key molecule in patients with DN and suggests that MK accelerates the intracellular signaling network evoked by hyperglycemia in DN. High glucose levels and diabetic substrates, such as glycation end products, affect mostly proximal renal tubular cells leading to tubular cell hypertrophy and the interstitial deposition of chemokines, cytokines like MK which cause inflammation and fibrosis of the tubules [30]. In line with these data, Brito et al. [31] have shown that the proximal tubular basement membrane is already thickened in normoalbuminuric patients with diabetes.

In the current study, we observed that the circulating levels of hs-CRP were significantly higher in diabetic children with microalbuminuria than the other two groups also in normoalbuminuric diabetic children in comparison to the healthy controls. Furthermore, the hsCRP levels were positively correlated with MK levels ($r=0.323$, $p=0.01$). These findings suggest that MK which is expressed in the proximal tubular epithelial cells of the kidney are thought to have a role in the pathophysiology of inflammation-related renal diseases[7]. MK is an endogenous inflammatory marker and a key molecule in the development of DN. It enhances both neutrophil and macrophage migrations in the tubulointerstitial regions, which were detrimental to kidneys [7]. Studies involving human biopsy specimens and animal models have reported that macrophage infiltration is a characteristic of DN, confirming the concept that inflammation plays a crucial role in the pathogenesis of DN [32].

In the current study, we showed that SBP SDS and DBP SDS were significantly higher in microalbuminuric group compared to normoalbuminuric group and the control group. Beside , DBP SDS was significantly higher in normoalbuminuric group compared with the control group. Hobo et al [33]. showed that SBP and mean BP were significantly higher in the Mdk+/+ mice than in the Mdk−/− mice in the remnant kidney model as a model of advanced renal injury. They also reported that MK up-regulated the pulmonary angiotensin converting enzyme (ACE) in the 5/6 renal ablation model of chronic kidney disease leading to increase BP. These data may suggest that MK plays an important role in the hypertensive response [34]. MK was induced in the lung endothelium by oxidative stress and subsequently up-regulated by ACE, which hydrolyzes Ang II to induce more oxidative stress, thus accelerating MK generation; this leads to a vicious cycle of positive feedback in the MK-Ang II pathway [33]. Kidney–lung interactions involving positive feedback between the renin-angiotensin system and MK might partly account for the pathogenesis of hypertension and kidney damage [35].

In our study, ROC curve analysis revealed that MK cutoff value of 1512 pg/ml was able to predict microalbuminuria in children with T1DM with a sensitivity of 96% and specificity of 92%. However, to our knowledge, this is the first study that evaluate MK levels in children with DN and defined a cut-off value. Therefore, further prospective studies are needed to validate this threshold.

Limitations of the study

- The small sample size is probably related to strict inclusion criteria of the studied cases .
- Single-center study limits generalizability.
- Due to the cross-sectional nature of the work., it is difficult to conclude whether higher MK levels are directly involved in the pathogenesis of DN complications or just association
- We were also unable to compare the examined parameters based on a histological diagnosis. It should be taken into consideration that the increased levels of MK might not only reflect renal tubular cell damage. There may also be an extrarenal source.
- Microalbuminuria was determined in spot urinary sample rather the 24-hr urine sample which is considered the standard method in determining microalbuminuria

Conclusions: The results of this study suggest that serum MK is a useful, novel, practical marker for the evaluation of renal involvement in children with T1DM, especially in normoalbuminuric children , However, new researches with a larger sample size and a prospective design are of great importance to clarify the predictive and pathophysiological role and significance of MK in the early phase and also in the progression of DN.

Data availability

The datasets generated and analyzed in the current study are available from the corresponding author upon reasonable request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The study protocol was approved by the Ethics Committee of Faculty of Medicine, Assiut Children University Hospital, Assiut, Egypt.

Informed consent Written informed consents were obtained from the parents of all participants.

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Table 1 Clinical and laboratory variables of diabetic patients (normoalbuminuria and microalbuminuria), and healthy control groups

	Microalbuminuric (Group 1) (n= 60)	Normalbuminuric (Group 2) (n= 60)	Healthy controls (Group 3) (n=60)	P value
Age	16.5±2.7	13.8±3.4	14.3±3.9	0.011
Male	32(53.3 %)	29(48.3%)	28(46.6%)	0.865
Diabetic duration(yr)	9.3±2.6#	6.2±1.4	-	**<0.001**
Insulin dose (IU/kg/day)	0.99 ± 0.26	0.89 ± 0.27	-	0.910
BMI SDS	0.68±0.8	0.64±0.4	0.63±0.5	0.172
SBP SDS	1.77 ± 0.4*#	1.3 ± 0.2*	0.63 ± 0.2	**<0.001**
DBP SDS	0.99 ± 0.3*#	0.4 ± 0.1*	0.32 ± 0.05	**<0.001**
Triglycerides(mg/dL)	183.9±35.8*#	126.7±16.9*	107.1±11.2	**<0.001**
Total cholesterol (mg/dL)	198.7±37.3*#	165.3±25.1*	127.3±15.3	**<0.001**
LDL cholesterol (mg/dL)	136±35.4*#	94.7±22.5*	87.7±12.2	**<0.001**
HDL cholesterol (mg/dL)	41.2±15.2*#	61.7 ± 15.9	69.8±13.2	**<0.001**
UACR (mg/g creatinine)	218.6±35.6*#	22.2±2.7*	10.3±2.7	**<0.001**
Hb1Ac (%)	9.2±1.3*#	7.8±0.4*	4.7±0.3	**<0.001**
hs-CRP (mg/L)	6.55±1.3*#	2.81±0.78*	0.39±0.11	**<0.001**
Serum creatinine (mg/dL)	0.71 ± 0.11	0.67 ± 0.12	0.65 ± 0.13	0.148
eGFR-Cr (mL/min/1.73 m2)	100.71 ± 27.88	103.09 ± 33.22	113.93 ± 30.21	0.208
Seum Midkine (pg/ml)	1847.2 ± 266.4*#	1158.4 ±1 57.6*	658.3 ± 79.3	**< 0.001**

Data are shown as mean ± standard deviation, unless otherwise specified.

* Significance versus control subjects. # microalbuminuric group vs. normalbuminuric group ;BMI: body mass index; SDS: standard deviation score; BP: blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; UACR: urinary albumin creatinine ratio; HbA1c: hemoglobin A1c; hs-CRP: high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate

Table2 . Correlations between the levels of midkine and demographic, clinical, and laboratory Variables.

Variable	r	p
Age (yr)	0.298	0.071
Disease duration (yr)	0.411	**0.006**
BMI SDS	−0.086	0.514
SBP SDS	0.465	**0.002**
DBP SDS	0.311	**0.002**
HbA1c(%)	0.373	**0.003**
hs-CRP (mg/L)	0.324	**0.041**
Total cholesterol (mg/dL)	0.298	0.071
Triglycerides (mg/dL)	0.254	0.052
HDL (mg/dL)	−0.145	0.320
LDL (mg/dL)	0.221	0.089
eGFR-Cr (mL/min/1.73 m^2)	0.105	0.415
UACR (mg/g creatinine)	0.754	**0.001**

BMI: body mass index; SDS: standard deviation score; BP: blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; UACR: urinary albumin creatinine ratio; HbA1c: hemoglobin A1c; hs-CRP: high-sensitivity C-reactive protein; eGFR, estimated glomerular filtration rate

Table 3. Multiple regression analysis of the relation of midkine to clinical and laboratory variables

Variable	Standardized coefficients	p-Value

Age (yr)	0.24	0.48
Disease duration (yr)	0.38	0.09
SBP SDS	0.22	0.86
DBP SDS	0.54	0.08
hs-CRP (mg/L)	0.61	**<0.001**
HbA1c (%)	0.66	**<0.001**
UACR (mg/g creatinine)	0.49	**<0.001**

BP: Blood pressure UACR: urinary albumin creatinine ratio; HbA1c: hemoglobin A1c; hs-CRP: high-sensitivity C-reactive protein;

Figure 1.