



Neutrophil gelatinase-associated lipocalin and Cathepsin L as early predictors of kidney dysfunction in children with type 1 diabetes

Lipokaina związana z żelatynazą neutrofilii i katepsyna L jako wczesne markery uszkodzenia nerek u dzieci z cukrzycą typu 1

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Abstract

Introduction: The aim of this study was to evaluate serum levels and urinary excretion of neutrophil-gelatinase associated lipocalin (respectively sNGAL and uNGAL) and urinary excretion of Cathepsin L (uCathL) in children with type 1 diabetes mellitus (DM1) who presented normoalbuminuria and the estimated glomerular filtration rate (eGFR) above 90 mL/min/1.73 m².

Material and methods: The study group consisted of 63 children with a diabetes duration of 5.16 ± 3.39 years. The degree of albuminuria was based on urine albumin-to-creatinine ratio (ACR), while eGFR was based on serum cystatin C. Glomerular hyperfiltration (GH) was defined as an eGFR value above 135 mL/min/1.73 m².

Results: Children with DM1 showed significantly higher concentrations of uNGAL, and lower sNGAL and uCathL. Significant changes of uNGAL and uCathL levels were even found in children without GH and with optimal glycaemic control ($HbA_{1c} < 7.5\%$). Positive correlations between uNGAL, ACR and eGFR were shown, as well as between uCathL and eGFR.

Conclusions: Significant changes in the concentration of markers of early kidney injury: sNGAL, uNGAL, and uCathL, can occur in children with DM1 and normoalbuminuria. The changes of uNGAL and uCathL can be even found in children without GH and with optimal glycaemic control. The earliest signs of diabetic kidney dysfunction seem to result from tubular damage. (*Endokrynol Pol* 2014; 65 (6): 479–484)

Key words: diabetic kidney disease; biomarkers; hyperfiltration; microalbuminuria

Streszczenie

Wstęp: Celem pracy była ocena stężenia w surowicy i w moczu markerów wczesnego uszkodzenia nerek, to jest lipokainy związanej z żelatynazą neutrofilii (odpowiednio sNGAL i uNGAL) oraz wydalania z moczem katepsyny L (uCathL) u dzieci z cukrzycą typu 1 (DM1) wykazujących normoalbuminurię i filtrację kłębuszkową (eGFR) powyżej 90 mL/min/1,73 m².

Materiał i metody: Grupę badaną stanowiło 63 dzieci ze średnim czasem trwania DM1 wynoszącym $5,16 \pm 3,39$ roku. Albuminurię oceniano za pomocą wskaźnika albuminowo-kreatyninowego (ACR). eGFR obliczano na podstawie stężenia cystatyny C. Hiperfiltrację kłębuszkową (GH) definiowano jako wartość eGFR > 135 mL/min/1,73 m².

Wyniki: U dzieci z DM1 w porównaniu do grupy kontrolnej wykazano znamienne wyższe stężenia uNGAL, niższe sNGAL i uCathL. Istotne zmiany stężeń uNGAL i uCathL stwierdzono już u dzieci bez GH i z optymalną kontrolą glikemii ($HbA_{1c} < 7,5\%$). Stwierdzono pozytywną zależność pomiędzy uNGAL, ACR i eGFR oraz pomiędzy uCathL i eGFR.

Wnioski: Istotne zmiany w stężeniu markerów wczesnego uszkodzenia nerek to jest sNGAL, uNGAL i uCathL mogą wystąpić u dzieci z DM1 i normoalbuminurią. Zmiany uNGAL i uCathL mogą wystąpić nawet u chorych bez GH oraz wykazujących optymalną kontrolę glikemii. Pierwsze objawy zaburzenia czynności nerek w przebiegu DM1 wydają się wynikać z uszkodzenia cewek nerkowych. (*Endokrynol Pol* 2014; 65 (6): 479–484)

Słowa kluczowe: dzieci z cukrzycą; biomarkery; hiperfiltracja; mikroalbuminuria

Introduction

A global increase in diabetes mellitus incidence of about 3% per year over the last decade has been recorded, with the most rapid increase in the youngest paediatric population in Europe [1]. One of the most dangerous

complications of the disease is diabetic kidney disease (DKD). Patients with type 1 diabetes (DM1) face a 20–50% probability of developing end stage renal disease (ESRD) [2]. There are several risk factors which are responsible for the development of DKD, including hyperglycaemia, reflected by high haemoglobin A_{1c}



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(HbA_{1c}) [3], but the predictors of DKD are only partly known.

Since the 1980s, microalbuminuria (MA) has been established as an early marker of progressive glomerular kidney disease in diabetes, starting at paediatric age [4–7]. However, some patients develop DKD without preceding MA and less than one third of patients with MA have typical glomerulopathy [8]. Some studies in human type 1 diabetes highlight the occurrence of glomerular hyperfiltration (GH) as a risk factor for nephropathy, which may precede an increase in albuminuria [9]. However, this remains controversial and the mechanisms responsible for GH have not been fully evaluated.

In recent years, neutrophil gelatinase-associated lipocalin (NGAL) has emerged in clinical and experimental nephrology as one of the most hopeful tubular biomarkers in the diagnostic field of acute and chronic renal diseases including glomerular diseases and DKD [10–15]. In patients with diabetic nephropathy, uNGAL predicts ESRD and even death [16]. However, the source and the time course of uNGAL concentration are not fully known.

Cathepsin L belongs to a subclass of cysteine proteases termed lysosomal cathepsins, which are primarily responsible for tissue turnover and protein breakdown in the kidney [17]. An analysis of kidney biopsy revealed increased activity of Cathepsin L in the glomeruli of patients with diabetic nephropathy. Induction of cytoplasmic Cathepsin L in glomeruli leads to the cleavage of dynamin, and the resulting reorganisation of the podocyte actin cytoskeleton and proteinuria [17].

With the present study, we aimed to evaluate serum levels and urinary excretion of NGAL and urinary excretion of uCathL in a cohort of children with type 1 diabetes mellitus who presented normoalbuminuria and estimated glomerular filtration rate (eGFR) above 90 mL/min/1.73 m².

Material and methods

Patient and control groups

The study group consisted of 63 children with DM1 (28 males and 35 females) with a mean age of 13.46 ± 2.95 years. The average treatment time was 5.16 ± 3.39 years. Children with infections, inflammatory states, proteinuria, pyuria or haematuria or any renal impairment were excluded to avoid potential confounding factors. All patients had an estimated glomerular filtration rate (eGFR) above 90 mL/min/1.73 m² according to the Filler formula based on serum cystatin C concentration, and urinary albumin excretion below 30 mg/g defined by the urine albumin-to-creatinine ratio (ACR) [18]. Screening for

albuminuria was performed three times in the first early morning urine sample over three months. The urine sample, collected on the same morning as the blood sample, was chosen for laboratory and statistical analysis. Long-term glycaemic control was based on haemoglobin A_{1c} (HbA_{1c}) levels [19]. Glycaemic controls were classified as follows: ideal — below 6.5% HbA_{1c}; optimal — below 7.5%; suboptimal — between 7.5 and 9.0%; and poor — above 9.0% HbA_{1c}. Glomerular hyperfiltration (GH) was defined as the sum of the mean and two standard deviations calculated for eGFR in the control group [20]. In this study, GH was defined as an eGFR value above 135 mL/min/1.73 m². There were no children with hypertension in the study group. None was overweight or obese.

The control group consisted of 22 healthy, age- and gender-matched children. The study protocol was approved by the local Ethics Committee; every participant and their parents/legal guardians gave fully informed consent to take part in the study. The study was conducted according to the principles listed in the Declaration of Helsinki.

NGAL and uCathL ELISA assay

Blood and urine samples were taken at the same time in the morning before food intake. All urine and blood specimens were used within three months after collection. NGAL was measured in serum and urine, using CircuLex Human NGAL/Lipocalin-2 ELISA Kit, while uCathL was measured in urine using Bender MedSystems ELISA Kit. Inter- and intra-assay coefficients of variations were 5–10%. To avoid differences in spot urine, all studied urinary markers were corrected for urinary creatinine. Common biochemical parameters were measured according to standard methods in a standard clinical laboratory.

The results of the biomarkers were normally distributed and parametric testing was used to compare all concentrations between groups (Student's *t*-test). Values that followed a normal distribution are expressed as mean and standard deviation. The relationship between two variables was assessed by Person correlation coefficient. The level of statistical significance was < 0.05 .

Results

The demographic and clinical data of the study group and controls are provided in Table I.

NGAL and uCathL studies

Children with DM1 showed significantly elevated uNGAL values compared to controls. sNGAL and uCathL were significantly lower in the study group compared to controls (Table II).

Table I. Demographic and clinical data of the study group and controls

Tabela I. Dane demograficzne i kliniczne grupy badanej i kontrolnej

	Study group Mean \pm SD	Controls Mean \pm SD	P
Age (years)	13.46 \pm 2.95	12.12 \pm 4.74	p = 0.451
Gender (female/male)	35/28	14/8	NA
RBMI (%)	104.72 \pm 13.41	101.81 \pm 16.57	p = 0.341
Treatment time (years)	5.16 \pm 3.39	NA	NA
ACR [mg/g]	10.8 \pm 9.84	9.13 \pm 5.25	p = 0.857
eGFR [mL/min/1.73 m ²]	116.89 \pm 23.18	110.11 \pm 12.41	p = 0.427
HbA _{1c} (%)	8.56 \pm 2.10	5.43 \pm 0.2	p < 0.001

NA — not applicable; SD — standard deviation; RBMI — relative body mass index

Table II. Differences in sNGAL, uNGAL and uCathL concentrations between study group and the controls

Tabela II. Porównanie stężeń sNGAL, uNGAL i uCathL grupy badanej do grupy kontrolnej

	Study group Mean \pm SD	Controls Mean \pm SD	p
sNGAL [ng/mL]	109.96 \pm 50.06	151.34 \pm 53.95	p < 0.001
uNGAL [ng/mg]	43.02 \pm 45.68	6.85 \pm 6.05	p < 0.001
uCathL [ng/mg]	4.32 \pm 1.61	5.18 \pm 0.95	p = 0.007

SD — standard deviation

Comparison of the study subgroups to controls depending on eGFR level (Table III)

GH was confirmed in 30% of children. In this subgroup, we found a significantly lower value of sNGAL and higher uNGAL as well as ACR and poor glycaemic control. uCathL level in these children did not differ compared to controls. Patients without GH differed from controls only in terms of uNGAL, uCathL and HbA_{1c}.

Comparison of the study subgroups to controls depending on HbA_{1c} level (Table IV)

uNGAL levels were significantly higher in children with optimal, suboptimal and poor glycaemic control, while sNGAL values were lower even in children with ideal glycaemic control. uCathL concentrations were significantly lower only in the subgroup with ideal and suboptimal glycaemic control. GFR and ACR values were significantly higher compared to controls only in children with poor glycaemic control.

Correlations

A positive correlation between uNGAL and ACR, eGFR and HbA_{1c} was found, as well as between uCathL and eGFR. The values of sNGAL showed a positive correlation only with WBC. The results are provided in Table V. HbA_{1c} levels revealed a positive correlation with eGFR

(r = 0.399; p = 0.014) and ACR (r = 0.268; p = 0.034). However, eGFR and ACR values were not significantly correlated.

In the subgroup with GH, a significant inverse correlation between uNGAL and sNGAL (r = -0.580; p = 0.048) was found. There was no correlation found between sNGAL and WBC in these patients.

In the subgroup without GH, a positive correlation between uNGAL and sNGAL (r = 0.402; p = 0.046), as well as between sNGAL and WBC (r = 0.712; p < 0.001), was found.

Discussion

It is well known that pathological albuminuria and proteinuria are the consequences of diabetes-induced glomerular damage. However, renal tubulointerstitium seems to play a major role in the genesis of DKD. Changes in the proximal tubule are important for the development of progressive diabetic kidney disease [21].

The results of the present study show elevated uNGAL and reduced sNGAL and uCathL in children with DM1 compared to controls. Increased uNGAL is in perfect accordance with the results reported elsewhere, showing similar tendencies for NGAL in patients with

Table III. Comparison of patients without and with glomerular hyperfiltration to the controls

Tabela III. Porównanie wybranych parametrów u pacjentów z i bez hiperfiltracji do grupy kontrolnej

Parameters	Study group		Controls
	eGFR level (mL/min/1.73 m ²); mean ± SD		Mean ± SD
	90 > eGFR ≤ 135; n = 44	> 135; n = 19; GH	
sNGAL [ng/mL]	129.57 ± 56.66	111.33 ± 48.86	
p	NS	p = 0.031	151.34 ± 53.95
uNGAL [ng/mg]	25.23 ± 37.14	56.41 ± 44.76	
p	p = 0.018	p < 0.001	5.56 ± 4.37
uCathL [ng/mg]	3.66 ± 0.37	5.16 ± 2.44	
p	p < 0.001	NS	5.18 ± 0.95
ACR [mg/g]	8.63 ± 5.42	14.27 ± 6.41	
p	NS	p = 0.021	9.13 ± 5.25
eGFR [mL/min/1.73 m ²]	103.24 ± 13.03	145.35 ± 9.06	
p	NS	p < 0.001	110.11 ± 12.41
HbA _{1c} (%)	7.87 ± 1.48	10.51 ± 2.76	
p	p < 0.001	p < 0.001	5.44 ± 0.21

GH — glomerular hyperfiltration; NS — not significant; p — confidence level; SD — standard deviation

Table IV. Comparison of the study subgroups to the controls depending on HbA_{1c} levelTabela IV. Porównanie grupy badanej do grupy kontrolnej w zależności od wartości HbA_{1c}

	HbA _{1c} level (%); mean ± SD				Controls Mean ± SD
	≤ 6.5; n = 9 6.02 ± 0.29% ideal glycaemic control	6.5 > HbA _{1c} ≤ 8; n = 21 7.21 ± 0.40% optimal glycaemic control	8 > HbA _{1c} ≤ 10; n = 20 8.98 ± 0.55% suboptimal glycaemic control	> 10; n = 13 11.86 ± 1.41% poor glycaemic control	
sNGAL [ng/mL]	106.62 ± 34.96	114.32 ± 59.11	120.43 ± 52.86	89.15 ± 34.56	
p	p = 0.026	p = 0.011	p = 0.044	p = 0.001	151.34 ± 53.95
uNGAL [ng/mg]	24.95 ± 38.35	40.34 ± 37.95	41.38 ± 45.88	62.41 ± 58.29	
p	NS	p = 0.013	p = 0.003	p < 0.001	6.85 ± 6.05
uCathL [ng/mg]	3.59 ± 0.72	4.53 ± 1.23	3.72 ± 0.30	5.41 ± 3.16	
p	p = 0.012	NS	p = 0.005	NS	5.18 ± 0.95
ACR [mg/g]	8.44 ± 5.69	9.09 ± 5.46	6.96 ± 3.66	16.83 ± 6.60	
p	NS	NS	NS	p = 0.004	9.13 ± 5.26
eGFR [mL/min/1.73 m ²]	109.09 ± 10.22	109.861 ± 23.79	110.53 ± 21.82	138.56 ± 20.26	
p	NS	NS	NS	p = 0.005	110.75 ± 12.41

NS — not significant; p — confidence level; SD — standard deviation

DM1 [11, 22, 23]. Considerable enzymuria, in particular uNGAL, in early stages of diabetes suggests tubular dysfunction before signs of glomerular damage become evident. In the studied patients, a significant rise of uNGAL occurred even in those without GH. Thus, the increase of uNGAL may reflect subclinical tubular

impairment, representing an earlier measurable index of renal distress. Urinary NGAL was related to the degree of ACR, even within a low range. This may indicate glomerular involvement in the pathogenesis of DKD. However, some authors claim that in diabetes, albumin excretion is caused by tubular dysfunction and

Table V. Statistical correlations between studied parameters in the diabetic group**Tabela V. Ocena zależności pomiędzy badanymi parametrami u dzieci z cukrzycą**

Parameters	ACR [mg/g]	eGFR [mL/min/1.73 m ²]	HbA _{1c} (%)	WBC(*10 ³ /mm ³)
sNGAL [ng/mL]	NS	NS	NS	r = 0.275 p = 0.030
uNGAL [ng/mg]	r = 0.365 p = 0.004	r = 0.331 p = 0.045	r = 0.287 p = 0.028	NS
uCathL [ng/mg]	NS	r = 0.501 p = 0.034	NS	NS

NA — not applicable; NS — not significant; r — correlation coefficient; p — confidence level

reduced lysosomal albumin degradation in proximal tubule cells [24].

Similar tendencies for NGAL in patients without the appearance of pathological albuminuria have been reported by other researchers [11, 22, 23].

In the present study, increased urinary loss of NGAL was accompanied by decreased serum NGAL concentration. This was previously described in an animal model of DM1 [22]. In human studies in type 2 DM (DM2), an elevated level of sNGAL was revealed; however, the studied patients showed normal or reduced eGFR [23]. Elevation of sNGAL was probably the consequence of glomerular damage and reduced eGFR. It might be also attributable to the increased lipocalin activity in patients with insulin resistance associated with obesity, which is characteristic for DM2 [25].

In our study, children were not obese and 30% of them showed GH. sNGAL values in children with GH were found to be significantly lower compared to controls. This observation may be indicative of the potential influence of hyperfiltration on sNGAL concentration; however, there is no similar data in the literature and some further research would have to be conducted on a larger study group.

There are only a few reports about cathepsins in DKD. Most of them refer to urinary Cathepsin B and show an increased loss in rats and in patients with DM2 [26, 27]. In rats, the activity of Cathepsins L and B was shown to be significantly decreased, which may be associated with renal hypertrophy in early diabetes [28]. Decreased activity of uCathL in diabetes may result from the inhibition by transforming growth factor beta (TGF-β) [24] and may lead to albumin loss resulting from the dysfunction of the degradation pathway and altered lysosomal processing in renal tubules. However, the potential importance of uCathL in DKD needs further investigation.

The results of our study confirm that the mechanism of early kidney damage in diabetes is not completely understood. They provide no clear answer to

the question as to which part of the kidney is damaged first: tubuli or glomeruli. The results of the present study have revealed that significantly elevated uNGAL, known to be a biomarker of tubular injury, and decreased uCathL levels, are found in children with DM1 without GH. These changes also precede the increase of ACR, hence, tubular injury seems to be earlier than the glomerular changes. On the other hand, in the early stage of kidney dysfunction in diabetes, we cannot exclude the impact of glomerular hyperfiltration on NGAL and Cath urinary loss as a positive correlation between uNGAL and eGFR was found in our study. This was suggested previously in an animal model of diabetic nephropathy [22]. Moreover, in patients with DM2, correlations between sNGAL, uNGAL and residual GFR were also found. These patients showed also a positive correlation between sNGAL and uNGAL [23]. In the present study, a positive correlation between sNGAL and uNGAL was found only in patients without GH. These children showed a correlation between sNGAL and WBC, which represents an important physiological source of this protein. This might suggest that the significant increase of uNGAL in this subgroup may result only from tubular injury. Interestingly, in patients with glomerular hyperfiltration, an inverse correlation between uNGAL and sNGAL was found, which may indicate an enhanced impact of GH on serum and urinary levels of studied biomarkers. The effect of hyperfiltration has also been expressed in uCathL, which showed a positive correlation with eGFR.

Taken together, these observations support the primary 'tubular hypothesis' of diabetic kidney disease. The results of our study showed increased albuminuria in patients with GH and in patients with poor glycaemic control compared to controls. Hence, albuminuria may be considered related to increased glomerular filtration and may reflect diabetic glomerular damage [29]. However, no correlation between ACR and eGFR was revealed.

The analysis of the relationship between glycaemic control and the analysed biomarkers revealed a positive correlation between HbA_{1c} and uNGAL, contrary to the reports of other authors [11, 23]. Significantly elevated ACR and eGFR values were only found in patients with poor glycaemic control; these results underpin the usefulness of NGAL and uCathL as early biomarkers of DKD.

This study has some limitations. Firstly, the study group is relatively small. Secondly, we did not evaluate the time-dependent changes of NGAL and uCathL levels. Thirdly, this was only one cross-sectional study and further studies on humans are needed to elucidate these points.

Conclusions

This study shows that some abnormalities suggesting early and subclinical kidney injury can be traced in children with DM1 and 'normal' renal function. Low-range albuminuria and normal eGFR do not exclude early kidney injury if defined as changes of sNGAL, uNGAL and uCathL concentrations. These changes may occur even in children without GH and with optimal glycaemic control. Glomerular hyperfiltration appears to enhance the observed changes. The earliest signs of kidney dysfunction manifested by changes of uNGAL and uCathL seem to result from tubular damage. Our study highlights the value of NGAL and uCathL as potential predictors for early DKD detection.

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