Original article

Association of serum and urinary neutrophil gelatinase-associated lipocalin (NGAL) levels with disease severity in patients with early-stage autosomal dominant polycystic kidney disease

Asocierea gelatinazei neutrofile serice şi urinare -asociate cu nivelele de lipocalin (NGAL) la pacienţii cu boală severă renală precoce şi rinichi polichistic autozomal dominant precoce

Gener Ismail[1*], Raluca Bobeica[2], Simona Ioanitescu[2], Roxana Jurubita[2]

1.Carol Davila University of Medicine and Pharmacy, Department of Internal Medicine-Nephrology, Fundeni Clinical Institute, Bucharest, Romania
2. Department of Internal Medicine-Nephrology, Fundeni Clinical Institute, Bucharest, Romania

Abstract

Introduction: Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent inherited renal disease. Monitoring disease progression will become more and more important with the introduction of new therapeutic agents. The use of kidney and cyst volumetry as markers of disease progression in patients with early stage ADPKD seems rational and evidence-based, but assessing these parameters is time consuming and expensive. Previous studies suggest that neutrophil gelatinase-associated lipocalin (NGAL) is a high-quality renal biomarker of acute tubular injury, while its applicability in ADPKD is not certain.Methods: Serum and urinary NGAL levels were assessed in 30 patients with early-stage ADPKD (GFR ≥90 ml/ minute/ 1.73 m² of body-surface area) and 30 healthy controls. Patients were further divided into two groups according to the cystic development assessed using magnetic resonance imaging: the group of patients with total kidney volume > 1500 cm³ (G1) and the group of patients with total kidney volume < 1500 cm³ (G2). Results: Serum and urinary NGAL levels were significantly higher in patients than in controls (sNGAL 159.5 ± 54.2 ng/ml vs. 53.1 ± 6.3 ng/ml, p < 0.05; uNGAL 113.5 ± 41.3 vs. 18.9 ± 5.9 ng/ml, p < 0.05) and these parameters were strongly correlated with the total renal volume (sNGAL/Total Renal Volume: r = 0.81, p < 0.0001; uNGAL/Total Renal Volume: r = 0.87, p < 0.0001).The median total renal volume was 2107.4 ± 442.7cm³ in the G1 group and 1121.9±253.9 cm³ in the G2 group. Subjects from the G1 group presented higher sNGAL and uNGAL levels compared to those from the other group (sNGAL: 181.5±55.6 vs. 137.5±44.3 ng/ml, p < 0.05; uNGAL: 137±33.1 vs. 89.9±35.3 ng/ml, p <

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[*]**Corresponding author:** Gener Ismail, Carol Davila University of Medicine and Pharmacy, Department of Internal Medicine-Nephrology, Fundeni Clinical Institute, Sos. Fundeni 258, Sector 2, Bucharest, Romania. Tel: 0722792429, E-mail: gener732000@yahoo.com

0.05). Conclusion: An accurate, non-invasive method for the regular assessment of disease progression in patients with early stage ADPKD would be very helpful in the management of these challenging patients. Our study suggests that serum and urinary NGAL levels might be useful as novel biomarkers in patients with ADPKD.

Keywords: NGAL, ADPKD, cyst growth, cystogenesis

Rezumat

Introducere: Boala polichistică renală autozomal dominantă (BPRAD) este cea mai frecventă afecţiune renală transmisă genetic. Monitorizarea progresiunii BPRAD devine extrem de importantă odată cu introducerea în ultimii ani a noi agenţi terapeutici. Există numeroase dovezi ştiinţifice ale utilităţii volumetriei renale CT sau RMN în monitorizarea progresiei BPRAD chiar şi în stadiile incipiente ale bolii, dar aceste tehnici imagistice necesită investiţii costisitoare ca timp si bani. Studii clinice efectuate anterior studiului nostru sugerează că lipocalina asociată neutrofilelor umane (NGAL) este un biomarker renal valoros pentru injuria tubulară acută, dar utilitatea sa în BPRAD este înca neclară. Material şi metode: Studiul nostru a evaluat nivelurile serice şi urinare ale NGAL la 30 de pacienţi cu BPRAD în stadiu incipient (RFG ≥90 ml pe minut pe 1.73 m² de suprafaţa corporală) şi la cei 30 voluntari sănatoşi din grupul de control. Pacienţii au fost împărţiţi în două grupuri în funcţie de rata de creştere a chisturilor (evaluată prin examen RMN), astfel: grupul pacienţilor cu volum renal total > 1500 cm³ (G1) si grupul pacienţilor cu volum renal total < 1500 cm³ (G2). Rezultate: Atât nivelurile serice cât şi cele urinare ale NGAL au fost semnificativ crescute în grupul pacienţilor cu BPRAD faţă de grupul de control (sNGAL 159.5 ± 54.2 ng/ml vs. 53.1 ± 6.3 ng/ml, p < 0.05; uNGAL 113.5 ± 41.3 vs. 18.9 ± 5.9 ng/ml, p < 0.05) şi s-a observat o strânsă corelaţie între aceşti parametri şi volumul renal total (sNGAL/ Volum Renal Total: r = 0.81, p < 0.0001; uNGAL/Volum Renal Total: r = 0.87, p < 0.0001).Volumul renal total median a fost de 2107.4 ± 442.7cm³ în grupul G1 şi de 1121.9±253.9 cm³ în grupul G2. Pacienţii din grupul G1 au prezentat niveluri mai mari ale sNGAL si uNGAL comparativ cu cei din grupul G2. (sNGAL: 181.5±55.6 vs. 137.5±44.3 ng/ml, p < 0.05; uNGAL: 137±33.1 vs. 89.9±35.3 ng/ml, p < 0.05). Concluzii: O metodă precisă, non-invazivă pentru monitorizarea progresiunii bolii la pacienţii cu BPRAD în stadii incipiente ar fi extrem de utilă pentru managementul acestor pacienţi care adesea sunt dificil de evaluat. Studiul nostru arată că nivelurile serice şi urinare ale NGAL pot reprezenta biomarkeri utili în aprecierea evoluţiei bolii la pacienţii cu BPRAD.

Cuvinte cheie: NGAL, boala polichistică renală autozomal dominantă

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is the most frequent inherited renal disease and mortality cause due to cystic disease in humans (1). It has an estimated prevalence of 0.07-0.20% of adults and represents one of the most frequent genetic disorders (2). ADPKD ranks third among causes of kidney disease in patients receiving chronic hemodialysis or renal transplant (3, 4).

In this disease, renal cysts are renal tubules dilatation at different levels, from the proximal segment to the collecting duct, with an epithelial lining. The development of renal cysts is focal and sporadic and these cysts increase in size and number with age (5).

During the last years possible therapeutic targets were identified and numerous clinical trials were initiated in line with progresses noted in understanding ADPKD pathogenesis. Under these circumstances, monitoring the progression of ADPKD especially at initial stages of disease when renal function is not yet severely impaired becomes more important.

Currently, changes in GFR are the gold standard for quantifying the progression rate in most chronic kidney diseases. However, due to the notable capability of intact nephrons to compensate for the loss of functional parenchyma, GFR measurement may fail to reveal threatening changes in early stages of kidney diseases. In advanced stages of the disease, progression could be monitored by following the GFR decrease,

but at that time therapeutic intervention might be too late to influence the outcome.

There is not much doubt that cyst growth is ultimately responsible for subsequent loss of glomerular filtration through direct (compression) and indirect (e.g. fibrosis) effects. Given the continuous rate of cyst growth during early ADPKD, kidney or cyst volume enlargement is an obvious surrogate endpoint for clinical trials at that stage.

ADPKD progression can be reliably assessed by MRI and CT imaging techniques and kidney volumetry. The use of kidney volumetry to investigate the effect of new investigational drugs in early stages of the disease seems rational and evidence-based, but these surrogate markers proved to be expensive and time consuming (6).

Major cellular defects involved in cystogenesis include increased proliferation and apoptosis, fluids and ions secretion, abnormal polarization of membrane proteins, changes of cell-matrix interaction and persistent fetal genes expression (7).

Findings of previous studies conducted on mice models of ADPKD have underlined that cyst development is also linked to tubulointerstitial abnormalities whereas cystic tubular epithelia express increased levels of tubular stress proteins, such as monocyte chemotactic protein 1 (MCP-1), osteopontin and neutrophil gelatinase-associated lipocalin (NGAL), suggesting they might play a role in cystogenesis (8).

Therefore the identification of serum or urinary biomarkers which allow to monitor the disease progression especially at initial stages of disease, having as starting point the recent discoveries related to cystogenesis, would be extremely helpful for evaluation of therapeutic agents efficacy.

NGAL or Lipocalin 2 is an important member of the lipocalin family. These proteins are a unique three-dimensional structure which allows lipocalins to act as efficient shuttles and transporters for different types of molecules as iron, retinoids, arachidonic acid, prostaglandins, fatty acids and steroids (9).

In humans, NGAL is expressed by different types of epithelial cells including renal tubular epithelial cells, in reply to numerous pathological conditions including ischemic, toxic or infectious renal damage (10).

The purpose of our study was therefore to evaluate the potential role of this protein as a biomarker for ADPKD progression assessment by analyzing its urinary and serum levels in patients with early-stage ADPKD.

Materials and Methods

Patients' characteristics

Our study was conducted on 30 patients with early-stage ADPKD (13 M, 17 F, mean age 40.2 ± 7.9 years) and a glomerular filtration rate (GFR) of ≥ 90 ml/ min. Mean level of serum creatinine was 0.96 ± 0.18 mg/dl and GFR (creatinine clearance calculated using the formula: urinary creatinine X urine volume/ 24h (l)/serum creatinine (mg/dl) X 1440min) was 106.9 ± 14.8 ml/min/1.73m^2). The diagnosis of ADPKD is established by specific ultrasound image or CT and positive family history. No patient received steroids, immunosuppressive medication. None of the patients had associated neoplasms, simultaneous infections and all of them signed the informed consent to enter the study which was approved by the local ethics committee. *Table 1* contains detailed clinical-biological characteristics of the patients.

The patients enrolled in the study were divided in two groups, depending on renal cysts development rate evaluated by magnetic resonance imaging (MRI). Patients having total renal volume > 1500 cm^3 (n=15) were included in high growth renal cyst rate group (G1), while patients having total renal volume < 1500 cm^3 (n=15) were included in slow growth renal cyst rate group (G2).

Control Group

The control group consisted of 30 healthy volunteers (13M, 17F; medium age 39.4 ± 7.7 years) having serum creatinine between 0.93 ± 0.15 mg/dl and a GFR medium value (creatinine

Table 1. Characteristics of 30 patients with ADPKD and 30 age- and sex-matched healthy controls

Variable	ADPKD	Healthy Controls
Age (y)	40.2 ± 7.9	39.4 ± 7.7
Men	13	13
BSA (m^2)	1.9 ± 0.11	1.89 ± 0.1
Plasma creatinine (mg/dl)	0.96 ± 0.18	0.93 ± 0.15
24-h urine volume(L/ 24h)	2.14 ± 0.23	2.11 ± 0.28
GFR (ml/min/1.73 m^2)	106.9 ± 14.8	117.2 ± 8
Total Renal Volume (cm^3)	1614.7 ± 613.9	-
sNGAL (ng/ ml)	159.5 ± 54.2[a]	53.1 ± 6.3
uNGAL (ng/ ml)	113.5 ± 41.3[b]	18.9 ± 5.9

BSA = body surface area; GFR = glomerular filtration rate; sNGAL = serum NGAL; uNGAL = urinary NGAL.
[a] $p < 0.05$ vs. Healthy controls; [b] $p < 0.05$ vs.Healthy controls

clearance calculated using formula: urinary creatinine X urine volume/ 24h (l)/serum creatinine (mg/dl) X 1440min) of 117.2 ± 8 ml/min/1.73 m^2.

Collection of Blood and Urine

Blood samples were collected in the morning on fasting patients. We also collected the second urine flow of the day. We put the blood specimens without delay into chilled blood collecting tubes containing potassium EDTA and the plasma was rapidly separated in a centrifuge.

Ten milliliters of fresh urine was centrifuged at 2,500 rpm for 10 min. All the urine and blood specimens were used immediately.

NGAL ELISA Assay

We used an ELISA commercial available kit (BioPorto Diagnostics, Denmark) to measure serum and urinary NGAL levels. The enzymatic reactions were read as quantitative results in an automatic microplate photometer (Biotek, USA). Our measurements were performed in a double blinded manner. We expressed NGAL levels as ng/ml.

Kidney volumetry

Patients underwent a standardized magnetic resonance imaging protocol of the abdomen without using an intravenous contrast substance. Renal volume was measured on T2-weighted coronal images.

Statistical Analyses

A statistical analysis of data was made using SPSS for Windows 17.0 (SPSS Inc, Chicago IL, USA). Continuous variables were expressed as mean ± SD. An unpaired two-tailed t test was used in comparing the two groups. In order to test correlations between variables, the Pearson's correlation coefficient was utilized. A p-value < 0.05 was considered statistically significant.

Results

Serum and Urinary NGAL Levels in ADPKD Patients and Control Subjects

In healthy subjects, serum NGAL levels (sNGAL) were 53.1 ± 6.3 ng/ml, while urinary NGAL levels (uNGAL) were 18.9 ± 5.9 ng/ml, values which fall within the normal range.

On the contrary, in ADPKD patients sNGAL levels were 159.5 ± 54.2 ng/ml and uNGAL levels were 113.5 ± 41.3 ng/ml having statistical significance with respect to controls (sNGAL, p < 0.05; uNGAL p < 0.05) (*Table 1*).

Correlation of NGAL levels and cystic mass growth rate

In our early-stage ADPKD patients, sNGAL and uNGAL levels were strongly cor-

Table 2. Clinical and biological characteristics of ADPKD patients.
Comparison between the group of patients with a total renal volume > 1500 cm^3 (G1) and the group of patients
with a total renal volume < 1500 cm^3 (G2)

Group	n	Age (years)	Sex M/F	sCr (mg/dl)	GFR (ml/min)	Total renal volume (cm^3)	sNGAL (ng/ml)	uNGAL (ng/ml)
G1	15	40.3±6.8	6/8	1.05±0.1	101.5±11.5	2107.4±442.7	181.5±55.6[a]	137±33.1[b]
G2	15	40.0±9.2	7/9	0.87±0.2	112.3±16.2	1121.9±253.9	137.5±44.3	89.9±35.3

sCr = Serum creatinine; GFR = glomerular filtration rate; sNGAL = serum NGAL; uNGAL = urinary NGAL.
[a] p < 0.05 vs. G2; [b] p < 0.05 vs.G2

Table 3. Correlation (r) between serum and urinary NGAL levels and cysts volume in patients with a total renal volume > 1500 cm^3 (G1) and patients with a total renal volume <1500 cm^3(G2)

	Total renal volume	
	Group G1	Group G2
sNGAL	r = 0.91[a]	r = 0.92[c]
uNGAL	r = 0.9[b]	r = 0.85[d]

[a] p < 0.0001; [b] p < 0.0001; [c] p < 0.0001; [d] p< 0.0001

related to total renal volume values (r = 0.81, p < 0.0001 for sNGAL and r = 0.87, p < 0.0001 for uNGAL).

No statistical difference was noticed in clinical and biological characteristics of patients with a total renal volume < 1500 cm^3 and of those having a total renal volume > 1500 cm^3. On the contrary, patients with a total renal volume > 1500 cm^3 (G1 group) showed significantly higher serum and urinary-NGAL levels than patients with a total renal volume < 1500 cm^3 (G2 group) (sNGAL: 181.5±55.6 vs. 137.5±44.3 ng/ml ng/ml, p < 0.05; uNGAL: 137±33.1 vs. 89.9±35.3 ng/ml, p < 0.05). Full data about the two groups are reported in *Table 2*.

In patients with a total renal volume > 1500 cm^3 (G1) serum NGAL levels (r = 0.91, p < 0.0001) and urinary NGAL levels (r = 0.9, p < 0.0001) correlated well with renal total volume (*Table 3*).

In patients with a total renal volume < 1500 cm^3 (G2) we found a good correlation between serum NGAL levels and total renal volume (r = 0.92, p < 0.0001) and also urinary

NGAL levels did correlate well with total renal volume (r =0.85, p < 0.0001) (*Table 3*).

Discussion

Autosomal dominant polycystic kidney disease is a common disorder, occurring in approximately 1 in every 400 to 1000 live births and also is the most common genetic cause of chronic kidney disease (11, 12). The majority of individuals with ADPKD eventually require renal replacement therapy (13).

The disease is a monogenic, autosomal dominant disorder with complete penetrance (14). The cysts development and enlargement is induced by the product of pathological gene polycystin1/polycystin2 (11, 13). While the precise genetic molecular mechanisms and biochemical mechanisms which initiate cysts development in ADPKD have not been clearly established, these mechanisms induce a clonal expansion of some partially differentiated epithelial cells characterized by dysregulation and

apoptosis of epithelial cells, a secretory pheno-type expression and a disturbance of cell-matrix interactions (4).

Even though synthesis of functionally abnormal polycystins is the main factor under-lying the dysregulation of tubular cells, which triggers cystogenesis, it is now evident that nu-merous other proteins participate in the disease development and progression.

In ADPKD, cysts formation occurs in all tubular segments. In a recent study, Meijer et al. show increased levels of both proximal (e.g. urin-ary β_2-microglobulin, urinary kidney injury mo-lecule-1 (KIM-1), N-acetyl-β-D-glucosamide (NAG) and NGAL) and distal tubular renal dam-age biomarkers (e.g. urinary heart-type fatty acid binding protein HFABP) in the urine of ADPKD patients versus healthy subjects (15).

NGAL is a 25kDa glycoprotein which forms covalent bindings with matrix metallopro-teinase-9. This protein seems to play different roles in kidney, only some of which have been well defined (16, 17). For example, it is known that it acts as an iron transporter, and thus appears to be involved in the embryonic and adult development of the renal epithelium through the induction of iron-dependent cellular signals with a consequent inhibition of the apoptotic processes (18). This in-hibition probably underlies the protective effect of NGAL against ischemic damage: increased serum and urinary levels of this protein have been ob-served in response to experimentally induced renal failure, and preventive administration of NGAL enhances the ability of the organ to sustain this type of stress (19). An acute increase in NGAL levels has also been observed in other experimental mod-els of tubular injury, including damage induced by nephrotoxic drugs and viral infections and, in hu-mans, after coronary angiography and cardiopul-monary bypass surgery (20, 21).

Viau et al. have demonstrated that NGAL gene inactivation inhibits tubular cells proliferation, leading to a significantly de-creased cyst growth in a murine model. The major source of NGAL production was identi-fied to be the cystic tubular epithelia. Also the authors have further identified Hypoxia-induct-ible factor 1α (Hif-1 α) as an essential mediator between EGFR and NGAL upregulation (22).

The findings of the present study are in accordance to those reported by Bolignano et al and clearly demonstrate that in ADPKD pa-tients, serum and urinary NGAL values are sig-nificantly higher than in control subjects (8).

Several studies identified a correlation between serum or urinary NGAL level and renal residual function indicating the levels of this pro-tein may in some way be influenced by the degree of the underlying altered renal function and even suggesting to use NGAL as a renal failure marker (8,17). Unlike these studies, we included in our study only patients with preserved renal function (GFR > 90 ml/min/1.73 m^2) in order to avoid the confounding effect of renal failure.

The aim of the present study was to test the hypothesis that NGAL could be used as a disease progression marker in ADPKD, espe-cially in the early phase when GFR is within normal limits and consequently is not useful for disease monitoring.

Ultrasound is less accurate to identify small changes in renal volume and is not suitable for very large kidneys. Using a long observation period with an average of 7.8 years of follow-up, however, Fick-Brosnahan et al. were able to demonstrate a significant inverse correlation between the rate of kidney volume growth and the rate of GFR decline (23). Computed tomography (CT) and MRI has been used to monitor polycyst-ic kidney volumes in several studies (24). Con-trast enhanced CT scanning or MRI or heavy-weighted unenhanced T2 MR images can reliably detect small cysts of 2 to 3 mm diameter (25). A concern related to the use of serial CT for disease monitoring in ADPKD is repeated exposure of young patients to ionizing radiation. Therefore, we used in our study magnetic resonance imaging (MRI) based volumetry which is more expensive but has the advantages of accuracy and lack of ex-posure to radiation.

Unlike other authors we were further divided our patients in two groups according to the cystic development assessed using magnetic resonance imaging: the group of patients with total kidney volume $> 1500\ cm^3$ (G1) and the group of patients with total kidney volume $< 1500\ cm^3$ (G2).The renal function (GFR) of the G2 group subjects was not significantly better than that of G1 group subjects.

In our study, serum and urinary levels of NGAL correlate with total kidney volume both in group G1 and in group G2. Nevertheless, in patients with a total kidney volume $>1500\ cm^3$, levels of sNGAL and uNGAL were significantly higher compared to those in patients with a total renal volume $<1500\ cm^3$, suggesting the involvement of this protein in the process of cyst formation and development, in a certain way.

These data suggest that there is a close link between NGAL and ADPKD. Additional evidence come to sustain this finding: first, NGAL-induced tubular development in *in vitro* assays in rats (26); second, NGAL infusion favored tubular regeneration after ischemic injury in mice (27).

In conclusion, our findings suggest that NGAL may act as a tubulogenic factor controlling cell growth. ADPKD progression can be reliably assessed by MR imaging techniques and kidney volumetry. These procedures are useful to assess the effect of new drugs in early stages of the disease, having still preserved, but are time consuming and expensive. It would be very helpful to have a precise and non- invasive method in order to evaluate repeatedly disease progression in patients with early stage ADPKD.

Our study suggests that serum and urinary NGAL may be an exciting biomarker for ADPKD progression assessment. More work is still necessary to demonstrate its NGAL suitability in routine clinical practice and to adjust the choice of discriminating cut-off values in different clinical situations and populations.

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