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ABSTRACT OF DOCTORAL THESIS

MOLECULAR BIOMARKERS OF DIAGNOSIS AND PROGNOSIS IN DIABETIC KIDNEY DISEASE

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1. INTRODUCTION

Diabetic kidney disease (DKD) is currently an important cause of morbidity and mortality in both type 1 and type 2 diabetes mellitus (DM).

From a clinical point of view, DKD is characterized by progressive renal impairment reflected by persistent albuminuria (> 300 mg/g creatinine), progressive decline in renal function (decreased glomerular filtration rate), to which cardiovascular complications are associated.

However, not all patients with DKD have persistent albuminuria. Thus, the terminology of non-albuminuric DKD is unanimously accepted, as microvascular complications that occur in patients with type 2 DM may be independent of the value of albuminuria. This fact is evidenced by biopsy studies which have shown that there are morphopathological changes that occur in the kidney, despite the absence of clinical signs of DKD.

This variability in clinical changes that occur in patients with type 2 DM suggests that the disease may be clinically underdiagnosed over a long period of time. A few serum and urinary biomarkers have been studied in DKD in order to detect the incipient changes that occur in kidneys, both at the glomerular and at the tubular level.

This thesis is a continuation of the studies prior to the doctoral thesis, in which I detected changes in the biomarkers of podocyte damage and of renal proximal tubule (PT) dysfunction, since the early stages of DKD. Thus, a lesser known theory in the pathogenesis of DKD is underlined, namely the early involvement of the PT. The PT dysfunction will lead to its inability to reabsorb filtered albumin, which will conduct to consecutive albuminuria.

Some researches in the current literature focused on the gene expression analysis, both within the glomerular and tubular compartment, emphasizing the importance of the gene expression profile in the pathogenesis of DKD. The possibility of quantifying the expression of certain RNAs, obtained by the transcription process from DNA, not only from renal tissues, but non-invasively, from biofluids (urine and serum) was an important step in understanding the pathogenesis of DKD. An example of non-invasive determination was one of the research topics in this doctoral thesis (Study 1), where I studied the involvement of urinary podocyte-associated messenger RNA in order to detect their implication in early DKD.

Along with a particular gene expression, epigenetic mechanisms, such as non-coding RNAs, (namely microRNAs-miRNAs), have proved their possible involvement in the pathogenesis of DKD (Study 2).

A topical aspect in my doctoral research has been the study of inflammatory mechanisms as possible triggers of renal impairment in type 2 DM (Study 3), as well as the interrelation between these inflammatory mechanisms and the epigenetic changes that occur early in DKD (Study 4).

Keywords**: albuminuria, diabetic kidney disease, microRNA, messenger RNA, interleukins, renal proximal tubule dysfunction, podocyte damage, podocyturia**

2. AIMS OF RESEARCH

The main aim of the current research was to identify a panel of molecular biomarkers that accurately describe the complex clinical and pathophysiological changes that occur in DKD, from the earliest stage, before the occurrence of albuminuria.

The specific objectives were such as the following:

1. The evaluation of urinary messenger RNA (mRNA) of podocyte-associated molecules in patients with type 2 DM, as well as their relation with urinary podocytes and biomarkers of podocyte damage, on the one hand, and with biomarkers of PT dysfunction, on the other hand.
2. The intervention of advanced glycation end-products (AGEs) in DKD and their relationship with biomarkers of podocyte damage, urinary podocytes, biomarkers of PT dysfunction and urinary mRNA of podocyte-associated molecules.
3. The investigation of a urinary microRNA (miRNA) panel in patients with type 2 DM in order to establish the correlations between changes in the levels of the studied miRNAs and the biomarkers of PT dysfunction, on the one hand, and those of podocyte damage, on the other hand.
4. The evaluation of the levels of serum and urinary pro-inflammatory cytokines (IL-1 alpha, IL-8, and IL-18) in relation to biomarkers of podocyte damage and of PT dysfunction.
5. The study of the relation of serum and urinary pro-inflammatory interleukins with the specific profiles of serum and urinary miRNAs in patients with DKD
6. The assessment of the predictive value of the studied biomarkers (mRNA, miRNA, pro-inflammatory cytokines, biomarkers of podocyte damage, podocyturia and biomarkers of PT dysfunction) in the diagnosis of DKD, since its early stage, before the occurrence of albuminuria.

3. PERSONAL RESEARCH

Study 1. Urinary podocyte-associated mRNA levels correlate with proximal tubule dysfunction in early diabetic nephropathy of type 2 diabetes mellitus

This study aimed to investigate the expression of certain urinary mRNAs of podocyte-associated molecules in DKD, namely nuclear factor kapa B (NFkB), the glomerular epithelial protein (GLEPP–1), CD2 associated protein (CD2AP), nephrin (NPHS1), ADAM metallopeptidase domain 10 (ADAM10), podocin (NPHS2), alpha-actinin-4, and to evaluate their relation with podocyte damage (expressed by biomarkers of podocyte damage and urinary excretion of podocytes), but also with renal PT dysfunction (expressed by PT-specific biomarkers). Also, another purpose of the study was to evaluate the urinary and plasma AGEs in DKD and their relation with urinary podocyte-associated mRNAs, as well as with biomarkers of podocyte damage and PT dysfunction.

The study enrolled 76 patients with type 2 DM, divided into 3 groups according to the level of albuminuria (28 patients were normoalbuminuric, 27 patients had microalbuminuria, 21 patients were macroalbuminuric, respectively), and 20 healthy patients, as the control group (group 4).

The ELISA (Enzyme-Linked Immunosorbent Assay) sandwich immunoassay technique was used to identify biomarkers of podocytes injury, namely urinary Vascular Endothelial Growth Factor (VEGF) and nephrinuria, biomarkers of PT dysfunction, such as urinary kidney injury molecule-1 (KIM-1), and urinary and plasma AGE peptides. The immunonephelometry technique was also used to determine albuminuria, urinary alpha-1 microglobulin, and serum cystatin C. The assessment of podocyturia was performed by cell culture of podocytes in vitro. Four steps were used to analyze the expression of the genes of interest, namely the nucleic acid extraction, quantification and assessment of RNA purity, reverse transcription and amplification (real-time polymerase chain reaction -RT-PCR).

The multivariable regression analysis showed that the expression of all 7 genes studied (NPHS1, NPHS2, alpha-actinin-4, CD2AP, ADAM10, GLEPP-1, NFkB) correlated directly with podocyturia, albuminuria, urinary alpha-1 microglobulin, urinary KIM-1, nephrinuria, VEGF, and indirectly with the estimated glomerular filtration rate (eGFR) ($p < 0.0001$, $R^2=0.808$; $p < 0.0001$, $R^2=0.825$; $p < 0.0001$, $R^2=0.805$; $p < 0.0001$, $R^2=0.663$; $p < 0.0001$, $R^2=0.726$; $p < 0.0001$, $R^2=0.720$; $p < 0.0001$, $R^2=0.724$).

Study 2. Deregulated profiles of urinary microRNA may explain podocyte injury and proximal tubule dysfunction in normoalbuminuric patients with type 2 diabetes mellitus

This study focused on the relation between a particular panel of urinary miRNAs (miRNA192, miRNA21, and miRNA124) and biomarkers of podocyte damage, such as urinary nephrin, urinary podocalyxin, urinary synaptopodin, on the one hand, and biomarkers of PT dysfunction, such as urinary KIM-1, urinary N-acetyl-beta-D-glucosaminidase (NAG), on the other hand. Another objective of the study was to demonstrate the early intervention of these urinary miRNAs in the diagnosis of DKD, before changes in albuminuria occur.

The study enrolled 68 patients with type 2 DM, divided into 3 groups according to the level of albuminuria (26 patients were normoalbuminuric, 24 patients had microalbuminuria, and 18 patients had macroalbuminuria, respectively) and 11 healthy patients, representing the control group (group 4).

The ELISA technique was used to assess biomarkers of podocyte damage (urinary nephrin, urinary synaptopodin, urinary podocalyxin) and to evaluate certain biomarkers of PT dysfunction (urinary KIM-1 and urinary NAG). Immunonephelometry was used to assess albuminuria, urinary alpha-1 microglobulin, and serum cystatin C. The steps for obtaining the studied miRNAs were represented by nucleic acid extraction, quantification and assessment of miRNA purity, reverse transcription and amplification (RT-PCR).

In univariable regression analysis, urinary miRNA21, miRNA124, and miRNA192 correlated with biomarkers of podocyte damage (urinary nephrin, urinary synaptopodin, urinary podocalyxin), biomarkers of PT dysfunction (urinary NAG, urinary KIM-1), as well as with the urinary albumin / creatinine (UACR) ratio and the eGFR.

In multivariable regression analysis, urinary miRNA192 correlated with urinary synaptopodin, urinary NAG, and eGFR ($p<0.0001$, $R^2=0.902$), urinary miRNA124 correlated with urinary synaptopodin, urinary NAG, UACR, and the eGFR ($p<0.0001$, $R^2=0.882$), and miRNA21 correlated with urinary podocalyxin, urinary NAG, UACR, and the eGFR ($p<0.0001$, $R^2=0.881$).

It should be noted that urinary miRNA192 expression was downregulated, while urinary miRNA21 and urinary miRNA124 expressions were upregulated.

Study 3. Pro-inflammatory cytokines are associated with podocyte damage and proximal tubule dysfunction in the early stage of diabetic kidney disease in type 2 diabetes mellitus patients

This study investigated the role of serum and urinary pro-inflammatory cytokines (IL-1 alpha, IL-8, IL-18) in the early stages of DKD, by evaluating them in relation to biomarkers of podocyte and PT dysfunction.

The study included 117 patients with type 2 DM (36 normoalbuminuric patients, 42 microalbuminuric patients, 39 macroalbuminuric patients, respectively), and 11 healthy subjects.

The ELISA sandwich method was used to assess biomarkers of podocyte damage (urinary nephrin, urinary synaptopodin and urinary podocalyxin), biomarkers of PT dysfunction (urinary KIM-1 and urinary NAG), as well as to evaluate serum and urinary interleukins (IL-1, IL-8, IL-18). Albuminuria and cystatin C were determined by immunonephelometry.

In the univariable regression analysis, the serum and urinary interleukins studied (IL-1 alpha, IL-8 and IL-18) correlated positively with the biomarkers of podocyte damage (urinary nephrin, urinary podocalyxin, urinary synaptopodin), with the biomarkers of PT dysfunction (urinary KIM-1, urinary NAG), as well as with albuminuria, and negatively with the eGFR.

In the multivariable regression analysis, urinary IL-1 alpha correlated directly with urinary podocalyxin and urinary NAG ($p<0.0001$, $R^2=0.57$), urinary IL-8 correlated positively with urinary synaptopodin, urinary KIM-1, urinary NAG, nephrinuria ($p<0.0001$, $R^2=0.67$), urinary IL-18 correlated directly with urinary synaptopodin, urinary NAG, and nephrinuria ($p<0.0001$, $R^2= 0.59$), serum IL-1 alpha correlated directly with nephrinuria, urinary synaptopodin, and urinary NAG ($p<0.0001$, $R^2=0.68$), serum IL-8 also correlated directly with urinary synaptopodin and urinary NAG ($p<0.0001$, $R^2=0.66$), and serum IL-18 correlated positively with urinary NAG, urinary KIM-1, and urinary podocalyxin ($p<0.0001$, $R^2=0.64$).

Study 4. Interleukins and miRNAs intervene in the early stages of diabetic kidney disease in type 2 diabetes mellitus patients

In this thesis, I analyzed the relationship of serum and urinary pro-inflammatory cytokines (IL-1 alpha, IL-8 and IL-18) with a certain profile of serum and urinary miRNAs, namely miRNA21p, miRNA124, miRNA125a, miRNA126, miRNA146a and miRNA192.

The study enrolled 117 patients with type 2 DM, who were divided into three groups, depending on the degree of albuminuria (36 normoalbuminuric

patients, 42 microalbuminuric patients, 39 macroalbuminuric patients, respectively), and 11 control subjects (group 4).

The laboratory techniques used were represented by the sandwich ELISA technique for the assessment of interleukins, the immunonephelometry for determining albuminuria and cystatin C, and the RT-PCR technique for determining miRNAs.

In the multivariable regression analysis, urinary ILs correlated with the studied urinary miRNA and the eGFR, such as follows: IL-1 alpha correlated directly with miRNA21 and 124, inversely with miRNA125a, miRNA192, and the eGFR ($p<0.0001$, $R^2=0.744$). IL-8 correlated directly with miRNA21 and miRNA124, and inversely with miRNA125a, miRNA126, miRNA146a, and the eGFR ($p <0.0001$, $R^2=0.726$). IL-18 correlated directly with miRNA21 and miRNA124, and inversely with miRNA146a, 192, and the eGFR ($p <0.0001$, $R^2=0.698$).

Regarding serum ILs, in multivariable regression analysis, serum IL-1 alpha correlated directly with serum miRNA124 and negatively with serum miRNA125a and miRNA192 ($p<0.0001$, $R^2=0.723$); serum IL-8 correlated inversely with serum miRNA146a and serum miRNA192 ($p <0.0001$, $R^2=0.743$); serum IL-18 correlated directly with miRNA21 and indirectly with serum miRNA125a and miRNA146a ($p<0.0001$, $R^2=0.636$).

4. CONCLUSIONS

1. The mRNA modifications and epigenetic factors are involved in DKD from an early stage

1.1. In patients with type 2 DM, urinary mRNAs of nephrin, podocin, CD2AP, ADAM 10, GLEPP-1, alpha-actinin-4, NFkB may be considered early markers of DKD as their expression was increased even since the normoalbuminuria stage. They also correlated with early urinary biomarkers of DKD, namely biomarkers of podocyte damage (nephrin and VEGF) and of PT dysfunction (alpha-1-microglobulin, KIM-1), as well as urinary excretion of podocytes.

1.2. Urinary and plasma AGEs may be involved in the alteration of the podocyte-associated mRNA expression (NPHS1, NPHS2, CD2AP, ADAM 10, GLEPP-1, alpha actinin-4, NFkB).

1.3. The studied urinary miRNA profile (miRNA21, miRNA124 and miRNA192) was associated with biomarkers of PT dysfunction (urinary NAG and urinary KIM-1), as well as with podocyte damage (urinary nephrin, urinary synaptopodin, urinary podocalyxin), from the normoalbuminuria stage, which indicates the early involvement of these miRNAs in DKD.

1.4. Urinary expression of miRNA192 is decreased in patients with DKD, while urinary expression of miRNA21 and miRNA124 is increased in

patients with DKD. This statement points to the fact that increased urinary expression of certain miRNAs (such as miRNA192) has a renal protective effect, while increased urinary expression of other miRNAs (such as miRNA21 and miRNA124) is associated with adverse renal effects.

2. Inflammatory molecules are involved in DKD from the earliest stages

2.1. The studied serum and urinary pro-inflammatory cytokines (IL-1 alpha, Il-8, and IL-18) are associated with biomarkers of PT dysfunction (urinary KIM-1, urinary NAG) and of podocyte damage (urinary nephrin, urinary synaptopodin, urinary podocalyxin), from the normoalbuminuria stage, a fact which demonstrates the role of inflammation in early DKD.

2.2. Each IL correlated differently with the studied biomarkers of podocyte damage (urinary nephrin, urinary synaptopodin and urinary podocalyxin), which can be explained by the fact that ILs may act through various mechanisms in certain areas of the podocyte structure (apical, junctional or basal domain).

3. There is a causal relationship between pro-inflammatory cytokines and epigenetic mechanisms in DKD in the early diagnosis of DKD

3.1. Serum and urinary ILs (IL-1 alpha, IL-8 and IL-18) are associated with the studied urinary and serum miRNAs (miRNA21, miRNA124, miRNA125a, miRNA126, miRNA146a, and miRNA192), since the normoalbuminuria stage.

3.2. The studied miRNAs could modulate the expression and activity of serum and urinary ILs, having a potential role in the development and progression of DKD.

3.3. Increased expressions of miRNA125a, miRNA126, miRNA146a, and miRNA192 may have a renoprotective effect.

LIST OF PUBLISHED PAPERS

1. Ligia Petrica, Sorin Ursoniu, Florica Gadalean, Adrian Vlad, Gheorghe Gluhovschi, Victor Dumitrascu, Daliborca Vlad, Cristina Gluhovschi, Silvia Velciov, Flaviu Bob, Petru Matusz, **Oana Milaș**, Alina Secara, Anca Simulescu, Roxana Popescu. Urinary podocyte-associated mRNA levels correlate with proximal tubule dysfunction in early diabetic nephropathy of type 2 diabetes mellitus. *Diabetol Metab Syndr* 2017;9:31. doi: 10.1186/s13098-017-0228-y
(ISI journal, **IF: 2,413**)

2. **Oana Milaș**, Florica Gadalean, Adrian Vlad, Victor Dumitrascu, Cristina Gluhovschi, Gheorghe Gluhovschi, Silvia Velciov, Roxana Popescu, Flaviu Bob, Petru Matusz, Agneta-Maria Pusztai, Octavian M Cretu, Alina Secara, Anca Simulescu, Sorin Ursoniu, Daliborca Vlad, Ligia Petrica. Deregulated profiles of urinary microRNAs may explain podocyte injury and proximal tubule dysfunction in normoalbuminuric patients with type 2 diabetes mellitus.*J Investig Med* 2018;66(4):747-754. doi: 10.1136/jim-2017-000556.
(ISI journal, **IF: 1,994**)

3. **Oana Milaș**, Florica Gadalean, Adrian Vlad, Victor Dumitrascu, Silvia Velciov, Cristina Gluhovschi, Flaviu Bob, Roxana Popescu, Sorin Ursoniu, Dragos Catalin Jianu, Petru Matusz, Agneta-Maria Pusztai, Alina Secara, Anca Simulescu, Maria Stefan, Mihaela Patruica, Flaviu Petrica, Daliborca Vlad, Ligia Petrica. Pro-inflammatory cytokines are associated with podocyte damage and proximal tubular dysfunction in the early stage of diabetic kidney disease in type 2 diabetes mellitus patients. *J Diabetes Complications* 2020;34(2):107479. doi: 10.1016/j.jdiacomp.2019.107479
(ISI journal, **IF: 2,684**)

4. Ligia Petrica, **Oana Milaș,** Mihaela Vlad, Adrian Vlad, Florica Gadalean, Victor Dumitrascu, Silvia Velciov, Cristina Gluhovschi, Flaviu Bob, Sorin Ursoniu, Dragos C Jianu, Petru Matusz, Agneta-Maria Pusztai, Octavian Cretu, Daniela Radu, Alina Secara, Anca Simulescu, Maria Stefan, Roxana Popescu, Daliborca Vlad. Interleukins and miRNAs intervene in the early stages of diabetic kidney disease in Type 2 diabetes mellitus patients. *Biomark Med* 2019;13(18):1577-1588. doi: 10.2217/bmm-2019-0124
(ISI journal, **IF: 2,268**)