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DOCTORAL (PhD) THESIS

EVALUATION OF THE ROLE OF PROINFLAMMATORY CYTOKINES AND URINARY GLYCOSYLATED PEPTIDES IN THE PREDICTION AND PROGRESSION OF DIABETIC CHRONIC KIDNEY DISEASE USING ADVANCED URINARY PROTEOMICS TECHNIQUES

ABSTRACT

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1. INTRODUCTION

DM is currently considered one of the oldest pathologies, causing a series of disabling complications, both short-term and long-term, and even death. Among the forms of DM known so far, DM 2 has the highest prevalence in the general population, the existing data on the mechanisms of appearance, and progression of this pathology undergoing a series of important changes over the years. DKD is currently considered the main cause for the severe deterioration of renal function, to the point when renal replacement techniques are required. Due to the fact that DM 2 is frequently late-diagnosed in most patients, they already have kidney damage at the time of diagnosis.

The diagnosis of renal impairment in DM 2 is established by evaluating two parameters: eGFR and albuminuria. These elements are used in medical practice in order to establish the diagnosis of DKD, to stratify the risk of progression, and establish possible therapeutic intervention. However, they have limitations, and are late detection elements of DKD, when the deterioration of renal function is already installed, and the progression of the disease has a fulminant character. Thus, new biomarkers are needed, for early detection of the disease, prior to the installation of irreversible kidney damage.

Proteins go through a multitude of processes in diabetic disease, prior to kidney damage, such as glycation, oxidation and nitration. There is a need for advanced proteomic techniques, that allow the detection of unique proteins or peptide fragments, which can become biomarkers of early detection in DKD.

miRNAs are a category of small, endogenous RNAs, that play an important regulatory role in modulating gene expression. They are involved in the initiation or progression of a variety of pathologies. Several types of miRNAs (21, 124, 125a, 126, 146a, 192) have been studied so far. They have shown various biological effects in the kidney, brain, and have the ability to modulate cellular and biochemical functions. In this way, they can initiate or stimulate the progression of complications in DM 2, including DKD, as well as cerebrovascular disease.

Pro-inflammatory cytokines play an important role in the development of DKD. They have direct renal effects and are capable to stimulate the expression of molecules that regulate local hemodynamics. They also influence extracellular matrix expression, affect GBM stability, stimulate oxidative stress, apoptosis and necrosis. Thus, the present study aimed to detect possible correlations between IL-6, IL-17 and certain miRNAs (21, 124, 146a, 192), as well as specific biomarkers of tubular and podocyte damage, in order to highlight the role of these cytokines in the pathogenesis of the disease.

The present study aimed to detect urinary proteins in patients with DM 2 and different degrees of renal impairment, using modern proteomic techniques. In this way, it demonstrated the importance of mass spectrometry in the early detection of DKD, and establised the basis for including these proteins in the clinician's detection routine, for an optimal control of the disease.

Subsequently, the remodeling process of the cerebral vessels, in the initial stages of DKD, was investigated. This phenomenona can be explained by the variability of miRNA expression in the two organs, kidneys and brain.

The involvement of certain interleukins, such as IL-6, IL-17, in the inflammatory mechanism of DKD, was then evaluated. Statistical correlations with specific miRNAs were also studied, as well as with known biomarkers of tubular and podocyte damage.

Key words: type 2 diabetes mellitus, urinary proteomics, albuminuria, inflammation, proximal tubule dysfunction, miRNAs.

2. PURPOSE OF THE RESEARCH

The present paper aimed to continue previous research, which identified relevant aspects related to the pathogenic mechanisms involved in glomerular and tubular injury in DKD. This paper also wanted to provide topical and clinically relevant perspectives, by highlighting new and early biomarkers, correlated with the initial stages of DKD. In this way, it may contribute to establishing new therapeutic approaches to the disease, in order to prevent or to slow down its evolution.

In accordance with these aspects, **the specific objectives** of this paper can be systematized as follows:

1. Use of advanced LC-MS / MS techniques in mass spectrometry, to detect unique proteins in DM 2 patients, which can be used as future biomarkers for early detection of DKD.
2. To evaluate the levels of particular miRNAs, through ELISA techniques and other complementary analysis tests, that can allow the assessment of possible connections between brain and kidney damage, in the initial stages of DKD.
3. To detect, using complex laboratory techniques, the involvement of certain proinflammatory cytokines, as well as certain miRNAs, in the early stages of DKD, by observing statistically significant correlations between these molecules and biomarkers of podocyte and tubular damage.

I. Specific urinary proteins, identified using modern proteomics techniques, can contribute to the discovery of new biomarkers in diabetic chronic kidney disease - a pilot study

This study aimed to implement an advanced proteomic platform for the detection of urinary proteins, in a group of subjects with DM 2 from Romania, who had different degrees of renal impairment and different stages of albuminuria, compared to a group of control subjects. The proteomic platform was tested and in-laboratory validated, using urine samples collected from both healthy and DM 2 subjects included in the study.

The results were then compared with existing data from the literature, in order to detect urinary proteins that can be classified as early biomarkers of renal impairment in DKD. Thus, a descriptive evaluation of the urinary proteins, identified in patients with DM 2, was performed, compared to the informations obtained in control subjects, using the results provided by advanced spectrometric analysis. A number of 557 complex human proteins were identified, using the MS/MS proteomics technique and then the SEQUEST spectrometry database search program, some detected only in patients` group.

In order to increase the quality of identification, to the initially identified proteins, three selection criteria were applied: the presence of at least three peptides unique to a protein group, the resulted sequence coverage of at least 30% at both test runs for at least one of the proteins identified in the patient group and a score of at least 10 for the identified peptides sequences (PSMs) for the proteins of each patient compared with those from the healthy control subjects. To obtain a better evaluation of the proteins that are in smaller quantities at the urinary level, those proteins that were found in high quantity were eliminated from the results. As so, immunoglobulins, transferrin, antitrypsin and haptoglobin were excluded. Although albumin is part of this group, urinary proteins were studied in type 2 DM patients, by reference to the degrees of albuminuria and so, albumin was taken into account.

Thus, from the total number of proteins identified, 31 proteins were selected, that meet these criteria. Subsequently, these proteins were classified according to their possible involvement in the pathogenesis of DKD, with implications in the glomerular, tubulointerstitial

and inflammatory mechanism of the disease. Thus, 71% of these proteins were correlated with the inflammatory mechanism, 16% with the tubulointerstitial mechanism and 13% with the glomerular mechanism, facts illustrated in figure 1.

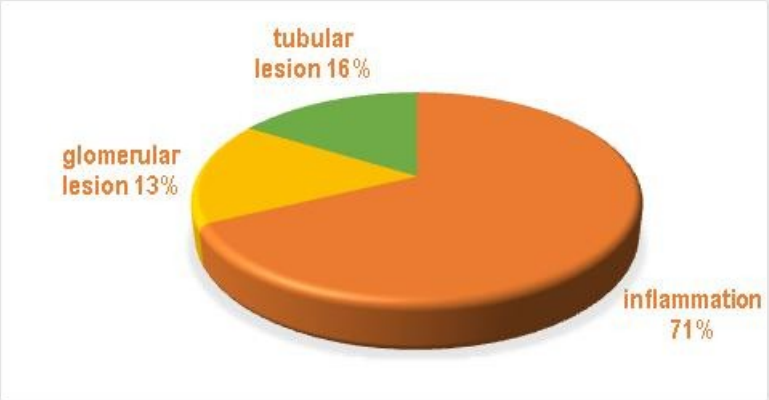

Figure 1. The percentage of proteins identified according to their pathogenic mechanisms in DKD

Out of the 31 proteins, selected based on the applied criteria, 5 proteins can be classified according to the possible mechanisms that are involved in the pathogenesis of DKD, as well as according to the level of albuminuria, as seen in Table 1.

Table 1 - Pathogenic mechanisms of selected proteins according to the level of albuminuria.

	Inflammation	Glomerular lesion	Tubular lesion
Micro-albuminuric	Hemoglobin subunit delta: human, gene name HBD, taxonomy 9606, SV=2, PE=1	Apolipoprotein A-I: human, gene name APOA1, taxonomy 9606, SV=1, PE=1	
Macro-albuminuric	Cytidine deaminase: human, gene name CDA, taxonomy 9606, SV=2, PE=1 Protein S100-A8: human, gene name S100A8, taxonomy 9606, SV=1, PE=1		Neutrophil gelatinase-associated lipocalin: human, gene name LCN2, taxonomy 9606, SV=2, PE=1

Conclusions:

1. Modern urinary proteomics techniques can be used to detect and evaluate urinary proteins that may play an important role in the pathogenesis of DKD.
2. It is possible to achieve, through modern proteomic platforms, the identification of urinary proteins that can be detected as possible biomarkers for early detection of DKD.
3. By generating a comparative proteomic profile between the patients` group and the control group, urinary proteins can be identified, that can be associated with the different mechanisms of DKD.
4. The use of an advanced proteomic platform is a powerful tool for rapid, sensitive and reliable discovery of protein biomarkers in human urine.
5. Designing and reporting a new method in mass spectrometry is a precondition for a favorable further evolution in the field of urinary proteomics.

II. miRNAs expression is associated with clinical changes due to vascular remodeling in the kidney and brain in patients with type 2 diabetes mellitus

The present study aimed to evaluate the expression of specific miRNAs found in the plasma and urine of patients with DM 2. These results were then compared with other known biomarkers of podocyte involvement and proximal tubule injury. Thus, the association between parameters of vascular remodeling in the kidneys, brain, and particular miRNA profiles in subjects with DM 2 was assessed. Taking into account previous studies, which studied the role of specific miRNAs in the kidneys in patients with DM 2, it was investigated whether those miRNAs may have effects in the brain also, due to the structural and functional similarities between the two organs. It has also been investigated whether certain miRNAs, involved in vascular remodeling in the brain, can cause effects in the kidneys.

The results obtained in this study showed much higher values of miRNAs expression in diabetic subjects compared to the control group; cerebrovascular changes were detected even in diabetic patients without pathological albuminuria. There were also modified values of podocyte and tubular damage biomarkers in the diabetic subjects. At the same time, there were statistically strong associations between renal, cerebro-vascular remodeling and specific miRNAs.

Multivariable regression analysis was used, and statistically significant correlations were identified between plasmatic, urinary levels of miRNAs 21, 124, 125a, 126, 146 a, 192, and ACR, eGFR, as well as with podocyte and tubular biomarkers (Table 2, 3).

Table 2 - Multivariable regression analysis using urinary levels of miRNAs

P	Variable	β Coef	P	95% CI	Prob (F)	R^2
U miRNA-21	Podocalixin/ creatinine	0.0005	0.0001	0.0002 – 0.0007	<0.00001	0.791
	NAG/creatinine	0.316	0.0001	0.023 – 0.039		
	ACR	0.0004	0.0001	0.0002 – 0.0007		
U miRNA-124	NAG/creatinine	0.062	0.0001	0.044 – 0.079	< 0.00001	0.749
	ACR	0.0004	0.001	0.0001 – 0.0007		
	eGFR	-0.009	0.0001	-0.014 – -0.004		
U miRNA-125a	Sinaptopodin/creatinine	-0.006	0.002	-0.002 – -0.010	< 0.00001	0.630
	NAG/creatinine	-0.054	0.0001	-0.031 – -0.077		
	eGFR	0.009	0.010	0.016 – 0.002		
U miRNA-126	Podocalixin / creatinine	-0.0005	0.0001	-0.0002 – -0.0007	< 0.00001	0.797
	NAG/creatinine	-0.020	0.0001	-0.013 – -0.028		
	ACR	-0.0005	0.0001	-0.0003 – -0.0007		
U miRNA-146a	Sinaptopodin/creatinine	-0.002	0.032	-0.005 – -0.0003	< 0.00001	0.801
	NAG/creatinine	-0.044	0.0001	-0.057 – -0.031		
	ACR	-0.0004	0.0001	-0.0007 – -0.0002		
	eGFR	0.005	0.009	0.001 – 0.009		
U miRNA-192	Sinaptopodin/creatinine	-0.003	0.007	-0.006 – -0.001	< 0.00001	0.811
	NAG/creatinine	-0.046	0.0001	-0.059 – -0.033		
	ACR	-0.0005	0.0001	-0.0007 – -0.0002		
	eGFR	0.004	0.020	0.0007 – 0.0089		

Legend: ACR- urinary albumin/creatinine ratio; eGFR – glomerular filtration rate; NAG/creatinine – urinary levels of N-acetyl-β-d-glucosaminidase/creatinine ratio; U miRNA- urinary levels of miRNA.

Table 3 – Multivariable regression analysis using plasmatic levels of miRNAs

P	Variable	β Coef	P	95% CI	Prob (F)	R^2
P miRNA-21	Podocalixin/creatinine	0.0005	0.0001	0.0002 – 0.0009	<0.00001	0.699
	NAG/creatinine	0.026	0.0001	0.016 – 0.037		
	ACR	0.0004	0.0001	0.0002 – 0.0007		
P miRNA-124	NAG/creatinine	0.020	0.006	0.034 – 0.006	< 0.00001	0.605
	ACR	0.0005	0.0001	0.0008 – 0.0003		
	KIM1/creatinine	0.0006	0.001	0.001 – 0.0002		
P miRNA-125a	Sinaptopodin/creatinine	-0.051	0.0001	-0.801 – -0.023	< 0.00001	0.482
	NAG/creatinine	-0.034	0.002	-0.055 – -0.013		
	Podocalixin/ creatinine	-0.006	0.002	-0.002 – -0.010		
	ACR	-0.0004	0.044	-0.0008 – -0.0001		
P miRNA-126	Podocalixin / creatinine	-0.004	0.001	-0.001 – -0.007	< 0.00001	0.562
	NAG/creatinine	-0.039	0.0001	-0.053 – -0.024		
	Sinaptopodin/creatinine	-0.033	0.001	-0.053 – -0.014		
	ACR	-0.0004	0.002	-0.0007 – -0.0001		
P miRNA-146a	NAG/creatinine	-0.048	0.0001	-0.065 – -0.030	< 0.00001	0.695
	ACR	-0.0005	0.0001	-0.0008 – -0.0002		
	eGFR	0.007	0.003	0.002 – 0.012		
P miRNA-192	Sinaptopodin/creatinine	-0.003	0.033	-0.0002 – -0.006	< 0.00001	0.655
	NAG/creatinine	-0.036	0.0001	-0.018 – -0.055		
	eGFR	0.011	0.0001	-0.018 – -0.055		

Legend: ACR- urinary albumin/creatinine ratio; eGFR – glomerular filtration rate; NAG/creatinine – urinary levels of N-acetyl-β-d-glucosaminidase/creatinine ratio; KIM- 1/creat – urinary levels of kidney injury molecule-1/creatinine ratio; P miRNA- plasmatic levels of miRNA.

In multivariable linear analysis, plasmatic levels of miRNA-21 and miRNA-192 correlated independently with RI-ICA, RI-MCA, BHI; miRNAs 124, 125a, 126, 146a indicated independent correlations with the same parameters of cerebral vascular remodeling, as well as with IMT-CCA (Table 4). Also, multivariable regression analysis indicated significant and independent correlations between neuro-sonological parameters (RI-ICA, RI-MCA, RI-CCA, BHI), ACR, eGFR, and biomarkers of tubular and podocyte lesions (Table 5).

Table 4 – Multivariable regression analysis of cerebral hemodynamic indices

P	Variabile	β Coef	P	95% CI	Prob (F)	R^2
P miRNA-21	RI-ICA	2.773	0.0001	1.470 – 4.076	<0.00001	0.441
	RI-MCA	1.836	0.004	3.080 – 0.592		
	BHI	-0.031	0.014	-0.056 – -0.006		
P miRNA-124	RI-ICA	-2.993	0.0001	-4.448 – -1.537	< 0.00001	0.405
	RI-MCA	-2.352	0.001	-0.962 – -3.741		
	BHI	0.041	0.004	0.013 – 0.069		
P miRNA-125a	RI-ICA	-1.624	0.010	-2.847 – -0.401	< 0.00001	0.421
	BHI	0.036	0.037	0.002 – 0.070		
P miRNA-126	RI-ICA	-3.228	0.001	-5.055 – -1.401	< 0.00001	0.408
	IMT-CCA	-1.829	0.010	-0.0457 – -3.201		
	BHI	0.038	0.008	0.010 – 0.065		
P miRNA-146a	RI-ICA	-1.403	0.010	-2.463 – -0.344	< 0.00001	0.528
	BHI	0.488	0.002	0.019 – 0.785		
P miRNA-192	RI-ICA	1.409	0.005	0.447 – 2.371	< 0.00001	0.583
	BHI	-0.049	0.0001	-0.076 – -0.022		

Legend: BHI- breath-holding index; RI- resistance index; CCA- common carotid artery; ICA- internal carotid artery; IMT- intima-media thickness; MCA- middle cerebral artery; P miRNA- plasmatic microRNA.

Table 5 - Multivariable regression analysis between cerebral hemodynamic parameters, biomarkers of podocyte, tubular impairment, eGFR and ACR

P	Variable	β Coef	P	95% CI	Prob (F)	R^2
RI-ICA	Sinaptopodin/creatinine	0.009	0.0001	0.005 – 0.013	<0.00001	0.795
	Podocalixin / creatinine	0.001	0.0001	0.001 – 0.0005		
	Nephrin / creatinine	0.011	0.024	0.001 – 0.022		
	eGFR	-0.003	0.0001	-0.004 – -0.002		
	Sinaptopodin/creatinine	0.014	0.0001	0.009 – 0.019	< 0.00001	0.730
	Podocalixin / creatinine	0.001	0.0001	0.002 – 0.001		
	eGFR	-0.005	0.0001	-0.006 – -0.004		
	ACR	0.006	0.032	0.001- 0.038		
IMT-CCA	Sinaptopodin/creatinine	0.014	0.0001	0.008 – 0.020	< 0.00001	0.785
	Podocalixin / creatinine	0.001	0.0001	0.002 – 0.0008		
	eGFR	-0.006	0.0001	-0.007 – -0.004		
	ACR	0.003	0.001	0.001- 0.008		
BHI	Sinaptopodin/creatinine	-0.259	0.010	-0.455 – -0.063	< 0.00001	0.656
	Podocalixin / creatinine	-0.030	0.022	-0.004 – -0.055		
	KIM 1 / creatinine	-0.006	0.040	-0.0003 – -0.013		
	NAG / creatinine	-0.194	0.023	-0.360 – -0.027		
	eGFR	0.173	0.0001	0.118 – 0.228		

Legend: BHI- breath-holding index; CCA- common carotid artery; ICA- internal carotid artery; IMT- intima-media thickness; MCA- middle cerebral artery; RI- resistance index; eGFR- estimated glomerular filtration rate; KIM-1/creat- urinary kidney injury molecule-1: creatinine ratio; NAG/creat - N-beta-D-acetylglucosaminidase: creatinine ratio.

The present study demonstrates that cerebrovascular changes are detected even in normoalbuminuric patients, who present modified levels of podocyte and tubular impairment biomarkers. At the same time, there are statistically strong associations between parameters of renal, cerebral vascular remodeling, and specific miRNAs.

The results obtained in the present study indicate the specific roles of miRNAs in cerebro-vascular remodeling, either by increasing vascular rigidity and stimulating atherosclerosis (miRNA-21, 192), or by providing vascular protection (miRNA-124, 125a, 126 and 146a). In fact, some miRNAs may have protective effects on the kidneys, and negative effects on the brain, or vice versa. This is the case with miRNA-192, which participates in reno-protective activities, while having negative effects on cerebral vascularity. On the other hand, miRNA-124 has opposite effects in both organs. While miRNA-21 is involved in producing negative renal and cerebral changes, miRNA-125a, 126 and 146 have protective effects at both levels.

Elevated urinary NAG levels were significantly associated with low IR values, which may suggest that NAG levels indicate incipient changes in brain microvascularization. At the same time, important correlations were detected between certain parameters associated with cerebral hemodynamics and certain biomarkers of tubular and podocyte involvement in DM 2, results independent of albuminuria and renal impairment.

The variability of expression and involvement of miRNAs in the brain and kidney, suggests the presence of cerebro-vascular remodeling in patients with DM 2 and normoalbuminuria, who still express high levels of biomarkers of podocyte and tubular damage. However, the results must be interpreted carefully, taking into account the complexity of the brain structure as well as the specific action of certain miRNAs.

Conclusions:

1. In patients with DM 2, there is an important association between the occurrence of vascular remodeling in the brain and kidney and the activity of certain miRNAs.

2. Changes in cerebral vessels are detected even in normoalbuminuric subjects, which may suggest early vascular brain damage in DKD.
3. miRNAs expression presents a high variability, as well as an important role, in both organs, brain and kidneys.
4. Detection of miRNAs profiles allows individualized treatment for each patient with DM 2, taking into account the most affected vascular territory in this pathology.

III. IL-6 and IL-17 proinflammatory cytokines correlate with specific miRNAs, as well as with podocyte and tubular impairment biomarkers, in the initial stages of DKD

The present study aimed to evaluate the presence of statistically significant associations between certain proinflammatory cytokines and specific miRNAs, known biomarkers of podocyte damage and tubular dysfunction, in patients with DM 2. In this way, the present study evaluated possible biomarkers of early renal impairment in DM 2, prior to the onset of albuminuria. This research started from the already known presumption, that certain proinflammatory molecules and miRNAs have modified levels and can influence each other in DKD. As so, their involvement in the initial stages of the pathogenesis of the disease was evaluated.

There was a higher expression of the proinflammatory cytokines in diabetic patients, compared to the control group. There were also important differences between the levels of miRNAs found in diabetic patients with different degrees of albuminuria, and the highest levels were detected in the macroalbuminuric patients.

Multivariable regression analysis was performed, and the results were adjusted to eliminate any factors that may interfere with the accuracy of the results, such as lipid profile, C-reactive protein, HbA1c. Regarding the serum IL levels, strong correlations were obtained between sIL-6 and synaptopodine, NAG, eGFR ($p < 0.0001$; $R2 = 0.805$), as well as between sIL-17 and synaptopodine, NAG, KIM-1, eGFR, ACR ($p < 0.0001$; $R2 = 0.941$) (Table 6). On the other hand, urinary levels of IL showed significant correlations as follows: between uIL-6 and synaptopodine, NAG, eGFR ($p < 0.0001$; $R2 = 0.889$), between uIL-17 and synaptopodine, nephrine, NAG, eGFR ($p < 0.0001$; $R2 = 0.905$), as can be seen in Table 7.

Table 6 – Multivariable regression analysis for serum levels of proinflammatory cytokines IL-6 and IL-17

P	Variable	β Coef	P	95% CI	Prob (F)	R^2
sIL-6	Constant	168.498	<0.0001	141.929 – 195.067	<0.00001	0.805
	NAG/creatinine	3.719	<0.0001	2.667 – 4.770	<0.00001	0.805
	Sinaptopodin/creatinine	0.378	<0.0001	0.230 – 0.526	<0.00001	0.805
	eGFR	-0.956	<0.0001	-1.210 – -0.701	<0.00001	0.805
sIL-17	Constant	195.421	<0.0001	133.934 – 256.908	<0.00001	0.941
	NAG/creatinine	6.896	<0.0001	4.401 – 9.392	<0.00001	0.941
	Sinaptopodin/creatinine	1.622	<0.0001	1.090 – 2.154	<0.00001	0.941
	KIM-1/creatinine	0.447	<0.0001	0.360 – 0.535	<0.00001	0.941
	eGFR	-1.267	<0.0001	-1.870 – -0.663	<0.00001	0.941
	ACR	0.028	0.005	0.008 – 0.047	<0.00001	0.941

Legend: ACR- urinary albumin/creatinine ratio; eGFR – glomerular filtration rate; KIM- 1/creat – urinary levels of kidney injury molecule-1/creatinine ratio; NAG/creat – urinary levels of N-acetyl-β-d-glucosaminidase/creatinine ratio; sIL- serum levels of interleukin.

Table 7 – Multivariable regression analysis for urinary levels of proinflammatory cytokines IL-6 and IL-17

P	Variable	β Coef	P	95% CI	Prob (F)	R^2
uIL-6	Constant	107.744	<0.0001	87.164 – 128.323	<0.00001	0.889
	NAG/creatinine	3.505	<0.0001	2.690 – 4.320	<0.00001	0.889
	Sinaptopodin/creatinine	0.523	<0.0001	0.408 – 0.637	<0.00001	0.889
	eGFR	-0.972	<0.0001	-1.169 – -0.774	<0.00001	0.889
uIL-17	Constant	218.858	<0.0001	175.297 – 262.419	<0.00001	0.905
	NAG/creatinine	6.992	<0.0001	5.282 – 8.703	<0.00001	0.905
	Sinaptopodin/creatinine	1.101	<0.0001	0.814 – 1.389	<0.00001	0.905
	Nephrin/creatinine	9.512	<0.0001	4.764 – 14.259	<0.00001	0.905
	eGFR	-1.604	<0.0001	-2.033 – -1.176	<0.00001	0.905

Legend: ACR- urinary albumin/creatinine ratio; eGFR – glomerular filtration rate; KIM- 1/creat – urinary levels of kidney injury molecule-1/creatinine ratio; NAG/creat – urinary levels of N-acetyl-β-d-glucosaminidase/creatinine ratio; uIL- urinary levels of interleukin.

Also, in multivariable regression analyze, statistically significant, positive associations were detected, between sIL-6 and miRNA-21, miRNA-192 and negative correlations with miRNA-124, miRNA-146a ($p < 0.0001$; $R2 = 0.862$); sIL-17 developed direct correlations with miRNA-21, miRNA-192 and negative correlations with miRNA-124 ($p < 0.0001$; $R2 = 0.745$). These aspects are observable in Table 8. Positive associations were also detected between uIL-6 and miRNA-21, respectively negative with miRNA-192 ($p < 0.0001$; $R2 = 0.886$); uIL-17 developed similarly direct correlations with miRNA-21 and negative correlations with miRNA-192 ($p < 0.0001$; $R2 = 0.860$). These aspects can be seen in Table 9.

Table 8 – Multivariable regression analysis for serum levels of proinflammatory cytokines IL-6 and IL-17 and serum levels of specific miRNAs

P	Variable	R^2	β Coef	P	Prob (F)	95% CI
sIL-6	Constant	0.862	90.041	<0.0001	<0.00001	43.114 – 136.969
	S miRNA-21	0.862	18.158	0.011	<0.00001	4.283 – 32.033
	S miRNA-124	0.862	-14.387	0.022	<0.00001	-26.667 – -2.106
	S miRNA-146a	0.862	-20.716	<0.0001	<0.00001	-31.485 – -9.947
	S miRNA-192	0.862	43.777	<0.0001	<0.00001	33.314 – 54.240
sIL-17	Constant	0.745	-7.410	0.942	<0.00001	-207.215 – 192.395
	S miRNA-21	0.745	234.441	<0.0001	<0.00001	159.427 – 309.456
	S miRNA-124	0.745	-122.922	<0.0001	<0.00001	-188.775 – -57.069
	S miRNA-192	0.745	65.994	0.016	<0.00001	12.598 – 119.390

Legend: P – parameter; siL- serum level of interleukin; S miRNA – serum levels of miRNA

Tabel 9 – Multivariable regression analysis for urinary levels of proinflammatory cytokines IL-6 and IL-17 and urinary levels of specific miRNAs

P	Variable	R^2	β Coef	P	Prob (F)	95% CI
uIL-6	Constant	0.886	93.413	0.003	<0.00001	32.142 – 154.683
	U miRNA-21	0.886	58.205	<0.0001	<0.00001	36.779 – 79.632
	U miRNA-192	0.886	-49.511	<0.0001	<0.00001	-65.234 – -33.788
uIL-17	Constant	0.860	133.808	0.085	<0.00001	-18.473 – 286.090
	U miRNA-21	0.860	162.914	<0.0001	<0.00001	109.661 – 216.168
	U miRNA-192	0.860	-83.845	<0.0001	<0.00001	-122.924 – -44.766

Legend: P – parameter; uiL- urinary levels of interleukin; U miRNA – urinary levels of miRNA

In the present study, the levels of podocyte impairment biomarkers were correlated with those of tubular dysfunction and all of them showed increased values in patients with DM 2, including those with normoalbuminuria. These observations indicate concomitant podocyte and tubular involvement in DKD, as well as the significant role of the proximal tubule in albumin reabsorption. IL-6 and IL-17 levels were elevated in all groups of diabetic patients and developed an upward trend with impaired renal function. It can be considered that there is a degree of chronic inflammation, which occurs as viable renal parenchyma is lost and, implicitly, renal function is reduced. Also, it can be considered that the important associations found between IL and miRNAs indicate the role of miRNAs in regulating IL expression and activity, and their involvment in the pathogenesis of DKD.

Conclusions:

1. In DKD, chronic inflammatory process plays a central role in the pathogenesis of the disease and elevated levels of the inflammatory cytokines IL-6 and IL-17, detected in this study, confirms this theory.
2. The strong positive correlations detected between these proinflammatory cytokines and certain biomarkers of podocyte damage (nephrine, podocalixin, synaptopodine) highlight a possible interconnection between the inflammatory mechanism of DM 2 and glomerular damage, in the early stages of DKD.
3. The positive correlations detected between the same inflammatory cytokines and certain known tubular damage biomarkers (KIM-1, NAG, alfa-1 microglobulin) demonstrate a possible interrelationship between the inflammatory mechanism of DM 2 and tubular involvement, in the subclinical stages of DKD.
4. miRNAs 21, 124, 146a, 192 play an important role in regulating IL activity and expression and are thus involved in the pathogenesis of early DKD.

ORIGINAL CONTRIBUTIONS

The original contributions brought in through this paper can be summarized as follows:

1. The present thesis is the only one, as far as is known, that tested, with the desire to implement in practice, a modern proteomic platform, capable of identifying urinary proteins in patiens with DM 2. These proteins were subsequently classified according to their possible involvement in one of the main triggering mechanisms of DKD and were classified according to the level of albuminuria. All these complex actions allowed, at the end of the study, to summarize a number of proteins that can be considered as future biomarkers of early renal impairment in DKD.
2. In type 2 DM patients, statistically significant associations were documented, concerning the parameters of vascular remodeling within the brain, kidney with plasmatic, urinary miRNA expression profiles, independently of albuminuria and the level of renal function decline, facts that have not been presented in other studies so far.
3. Important connections were identified between the levels of certain interleukins and certain biomarkers of podocyte and tubular damage, as well as specific miRNAs, facts that have not been studied so far in this form and which allowed to draw conclusions concerning the early pathogenic mechanisms of DKD.

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