VICTOR BABEȘ UNIVERSITY OF MEDICINE
AND PHARMACY FROM TIMIȘOARA
FACULTY OF MEDICINE
DEPARTMENT OF NEPHROLOGY

BALINT P. LAVINIA

PhD THESIS

IDENTIFICATION AND CHARACTERIZATION OF GUT DERIVED METABOLITES IN DIABETIC KIDNEY DISEASE AND THEIR RELATIONSHIP WITH PODOCYTE, PROXIMAL TUBULE AND RENAL AND CEREBROVASCULAR ENDOTHELIAL MARKERS. A STUDY REALIZED BY ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED WITH ELECTROSPRAY IONIZATION QUADRUPOLE-TIME OF FLIGHT-MASS SPECTROMETRY

A B S T R A C T

Scientific Coordinator
Prof. LIGIA PETRICA, MD, PhD, Habil dr.

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INTRODUCTION

Type 2 diabetes mellitus (T2DM) leads to renal and cerebral damage due to endothelial dysfunction. Diabetic kidney disease (DKD) and cerebral small vessel disease (CSVD) are concurrent and severe complications of T2DM that can result in dementia, and elevated mortality rates by the occurrence of end stage renal disease (ESRD). The current DKD diagnostic methods are based on serum creatinine and urinary albumin-to-creatinine ratio, but the increasing prevalence of end-stage renal disease (ESRD) secondary to DKD, highlights the insufficiency of these markers to detect early disease.

In this sophisticated millieu created by T2DM, the need to identify novel biomarkers of early DKD and CSVD becomes imperative. Thus, the study of gut microbiota is gaining appreciation as it captures a vast collection of derived metabolites. Metabolomic techniques have emerged as versatile methods for profiling the metabolic flow within an individual, providing crucial information about metabolic pathways and disrupted homeostasis related to diverse disease scenarios. The evaluation of metabolites originating from gut microbiota using these techniques, may potentially be fundamental in the timely identification and the development of individualized treatments for patients suffering from T2DM and associated complications - DKD and CSVD.

INTROSPECTION ON RECENT DATA FROM THE LITERATURE

1. **THE CONCEPT OF TYPE 2 DIABETES MELLITUS AND RELATED COMPLICATIONS: DIABETIC KIDNEY DISEASE AND CEREBRAL SMALL VESSEL DISEASE**

Diabetes mellitus (DM) is a complex and often misdiagnosed disease, accounting for 90% to 95% of all cases globally. T2DM is the first cause of chronic kidney disease (CKD), causing 10% of deaths due to ESRD. The diagnosis of this condition may be determined by: hyperglycemia > 200 mg/dl at any time of the day and/or levels that exceed 126 mg/dl under fasting conditions, or hemoglobin A1c (HbA1c) levels > 6.5%.

As proposed by The Kidney Disease: Improving Global Outcomes (KDIGO) Clinical Practice Guideline for the Evaluation and Management of CKD is diagnosed either by the presence of renal structural damage (uACR exceeding 30 mg/g, changes in urinary sediment with the presence of hematuria, renal tubular disorders, and histopatological modifications may indicate) or by the presence of renal functional decline (eGFR of 60 ml/min/1.73 m^2 or less).

DKD is a dominant, microvascular complication, occuring in 20% to 40% of patients suffering from T2DM. As proposed by KDIGO work group, in a T2DM patient, moderately increased albuminuria and/or a decline in eGFR under 60ml/min/1.73 m^2, over a persistent period of 90 days, on repeated determinations, are the main pointers of DKD onset.

CSVD is not fully considered a microvascular complication of T2DM, however the study of its pathophysiology may provide answers, as the brain vessels share similar feautures with the renal vessels. The hyperglicemic milieu, by impaired autoregulation and neurovascular uncoupling, disrupts BBB integrity and determines the onset of CSVD. Its diagnosis is possible only using magnetic resonance imaging (MRI) or cerebrovascular Doppler Ultrasound, but these techniques fail to detect early disease.

2. THE GUT-RENAL CEREBRAL AXIS IN THE COMPLEX CONTEXT OF DIABETIC KIDNEY DISEASE

The human gastrointestinal tract hosts trilions of bacteria which act in a simbiotic fashion with the host as they maintain intestinal integrity and provide local nutrients. Prolonged hyperglicemia disrupts microbiota homeostasis, leads to an increased intestinal permeability and to a chronic inflammatory state, and ultimately determines intestinal dysbiosis. Intestinal dysbiosis in T2DM is characterized by low butyrate producing bacteria.

Prolonged hyperglicemia determines endothelial dysfunction by increased oxidative stress, aberrant production pro-inflammatory and pro-thrombotic factors. The reduction of heparan sulfate synthesis results in defective angiogenesis, dysruption of glycocalyx, enlargement of the fenestration and enhanced vascular permeability which determine urinary albumin loss. In addition, T2DM is characterized by podocyte apoptosis, excessive extracellular matrix (ECM) production by mesangial cells, and glomerular basement membrane (GBM) thickening. Proximal tubular cells express mitochondrial dysfunction, increased metabolic activity and reduced oxygen delivery, which result in cell apoptosis, with subsequent tubular atrophy and interstitial fibrosis.

The brain-kidney axis in the setting of DKD is characterized by defective glycolysis, increased oxidative stress and endothelial cell dysfunction. This translates into disrupted blood-brain barrier (BBB) integrity and increased passage of neurotoxins into the extravascular compartment. The gut-kidney axis contribues to DKD progression by increased intestinal permeability and increased passage of uremic toxins into the bloodstream which impact all renal structures (podocytes, endothelial, tubular, and mesangial cells). The gut has a complex connection with the brain by producing various neurotransmitters, whereas the brain influences microbial metabolism or colonization by the production of diverse chemical compounds, Thus, it may be hipothesized that gut dysbiosis may initiate cerebrovascular disease.

3. TRADITIONAL BIOMARKERS OF EARLY DIABETIC KIDNEY DISEASE DIAGNOSIS

With regard to endothelial dysfunction, investigations were previously conducted focusing on vasoactive molecules (endothelin-1, prostacyclin-2, and NO), inflammatory markers (interleukin (IL) β1, IL-6, and tumor necrosis factor α (TNF-α)), and drivers of oxidative stress (AGEs and ROS). Subsequently, attention was redirected towards the examination of molecules that are released by endothelial cells in response to injury, including monocyte chemoattractant protein (MCP-1) and intercellular adhesion molecule 1 (ICAM-1).

The most studied markers of podocyte damage were: nephrin, which modulates slit pore diaphragm structure; podocalyxin (PDX) which controls glomerular permeability; and synaptopodin (STP) which modulates actin cytoskeleton contractility.

The studied markers of proximal tubular damage associated with DKD were as it follows: kidney injury molecule (KIM-1) which is released in a context of proximal tubule injury even before the appearance of albuminuria; N-acetyl-β-D-glucosaminidase (NAG) reflects lysosomal activity and is a predictor of albuminuria; and neutrophil gelatinase-associated lipocalin (NGAL) is a biomarker rapidly released in the setting of severe tubular injury.

4. NOVEL INSIGHTS INTO METHODS OF BIOMARKER DISCOVERY

The progressive nature of DKD remains an issue despite scientists attempts to establish more accurate early detection methods. Omic sciences are utilized in systems medicine to investigate metabolic pathways and metabolite flow in various disease scenarios. These may lead to the development of novel therapeutic strategies, in order to limit disease progression.

Lipidomics brings substantial contributions to early DKD development. For example, the extent of sialylation of glycosphingolipids was identified to be correlated with the severity of DKD, even during the initial stages. By proteomic techniques, it was found a network of 273 peptides refered to as CKD273, aimed to function as a "liquid biopsy" for the early DKD detection. By using transcriptomic technqiues it was discovered that long-noncoding ribonucleic acid (lncRNA) is involved in the early stages of DKD through the regulation of micro ribonucleic acid (miRNA) expression.

Metabolomics provides the qualitative and quantitative assessment of a multidimensional metabolic feedback to pathophysiological or epigenetic stressors in order to build up a personalized profile that matches the exclusiveness of a fingerprint for each individual. This technique evolved from nuclear magnetic resonance (NMR) to ultra-high-performance liquid chromatography (UHPLC) coupled with mass-spectrometry (MS) which has a greater sensitivity for biomarker discovery. The UHPLC-MS technique consists of two major steps, such as: the untargeted (qualitative), multivariate and univariate analysis and the targeted (quantitative) analysis of metabolites with proven biomarker potential.

5. CLASSES OF METABOLITES EXPRESSING BIOMARKER POTENTIAL IN DIABETIC KIDNEY DISEASE AND CEREBRAL SMALL VESSEL DISEASE

Free amino acids (AAs) derive from proteic compunds and their homeostasis is closely regulated by the gut and the kidney. L-arginine is a precursor of NO and dimethylarginines. The clinical context of DKD was found to be associated with increased levels of ADMA and decreased levels of L-arginine. Moreover, L-arginine was also found to be linked to the degree of cerebrovascular endothelial dysfunction. Phenylalanine is an aromatic AA and a precursor of a variety of metabolites (tyrosine, catecholamines, etc.). In parallel, tyrosine is a semi-aromatic AA and a substrate for uremic toxin production. In humans, phenylalanine and tyrosine were found to be associated with T2DM macrovascular and microvascular related complications. The metabolism of the AA tryptophan follows three major routes of metabolism: kynurenine, serotonine and indole. Enhanced kynunrenine levels were found to be protective against T2DM related cognitive impairment; increased plasma serotonine may mirror incipient renal damage; and an indole dysregulated pathway may impact glucose metabolism and enhance oxidative stress. Thus, the study of AA metabolism may bring novel insights with regard to DKD development.

Uremic toxins derive from gut microbiota, enter the bloodstream and undergo sulfation in the liver. The clearance of these metabolites by renal tubular secretion via organic anion transporters (OATs) suggests their role in renal disorders. For example, hippuric acid was found to be decreased in the serum of patients suffering from DKD and non-DKD. In the BBB hippuric acid blocks OATs and determines the accumulation of other uremic toxins, such as indoxyl sulfate. Indoxyl sulfate was found to be a generator of renal endothelial dysfunction via aryl hydrocarbon receptors (AhR). It also determines the apoptosis of cerebrovascular endothelial cells and induces oxidative stress in microglia and astrocytes. In parallel, phenyl sulfate was found to be associated with mitochondrial

dysfunction and enhanced oxidative stress in podocytes and proximal tubular cells, whereas p-cresyl sulfate was found to induce renal fibrosis.

Acylcarnitines are fatty acid (FA) transport mediators into mitochondria, which are meant to undergo β-oxidation and create energy as ATP molecules. An increase in these molecules indicates mitochondrial dysfunction, and proximal tubule dysfunction in DKD. In DKD, carbohydrate metabolism is altered, switching from the glycolysis normal process to polyol and hexosamine pathways. These reactions are crucial because their end-products exert deleterious effects on endothelial cells, thereby causing damage to the renal filter or BBB, or because they imprint dysfunctionality to podocytes and tubular cells. Therefore, they are worth a special consideration in terms of DKD development.

Antioxidants, such as ascorbic acid and α-tocopherol are able to decrease serum creatinine levels in DKD. Furthermore, the retinoic acid signalling pathway seems to be of extreme significance in DKD. All-trans retinoic acid (ATRA) administration was found to inhibit glomerular and tubular inflammation. Moreover, the administration of ATRA has shown positive effects on post-cerebrovascular infarction outocmes, reduction in infarct size and amelioration of BBB permeability. These studies suggest the potential involvement of ATRA in the pathogenesis of DKD and CSVD.

PERSONAL RESEARCH AND CONTRIBUTIONS

T2DM progression continuously poses challenges because of ESRD development and limited therapeutic approaches. Molecular studies have explored the mechanisms contributing to DKD. Metabolomics has emerged as a powerful tool for understanding medical conditions and drug interactions. However, metabolite analysis alone does not establish a causal relationship and must be complemented with other markers. The Enzyme-Linked Immunosorbent Assay (ELISA) is a widely used immunoassay method for identifying antibodies and antigens. Doppler ultrasound assesses cerebrovascular dynamics and blood flow and provides valuable data regarding cerebrovascular autoregulation and vascular remodeling.

The first study aimed to identify distinct metabolite pathways, from serum and urine of patients with DKD compared to controls using untargeted multivariate methods. Univariate analysis revealed the metabolites that draw differences between DKD subgroups and controls. The results displayed that retinoic acid signaling pathway and nitrogen metabolic pathway were linked to DKD development before albuminuria onset.

The second study analyzed metabolites with biomarker potential, from serum and urine, in a targeted manner, specifically those found significant in the first study, with a special attention to normal to mildly increased albuminuria subgroup. The results revealed a correlation between serum arginine, dimethylarginine, hippuric acid, indoxyl sulfate, butenoylcarnitine, sorbitol, and urine p-cresyl sulfate levels and albuminuria. These metabolites were dysregulated prior to albuminuria onset.

The third study investigated the correlation between serum metabolites and indicators of renal and cerebrovascular endothelial damage, and urine metabolites with

podocyte dysfunction, and proximal tubule damage. The results indicated that in serum: arginine, butenoylcarnitine, and indoxyl sulfate are associated with renal endothelial dysfunction and cerebrovascular remodeling, whereas sorbitol is a biomarker of BBB dyfunction. In urine, butenoylcarnitine and indoxyl sulfate were linked with podocytopathy in the course of DKD, whereas p-cresyl sulfate is linked with proximal tubule dysfunction.

6. UNTARGETED MULTIVARIATE AND UNIVARIATE METABOLOMICS PROVIDES NEW INSIGHTS RELATED TO THE GUT-RENAL-CEREBRAL AXIS IN PATIENTS WITH EARLY DKD AND T2DM

6.1. AIMS

The aims of the first study were: to identify novel potential gut-derived biomarkers that may contribute to the development of early DKD; to indicate the metabolic pathways in which these biomarkers are involved; to emphasize how closely the early processes of DKD and CSVD are, which include the altered metabolic pathways and their relationship with endothelial dysfunction.

6.2. MATERIALS AND METHODS

The selection of patients. This study had a pilot and a cross-sectional design and included 110 participants (90 DKD patients – P group; and 20 controls – C group) monitored periodically in the County Emergency Hospital of Timisoara and at the general physician's office, respectively. The inclusion criteria were as it follows: DM duration above 5 years, HbA1c < 10%. All patients were treated with angiotensin 2 converting enzyme inhibitors/angiotensin 2 receptor blockers, statins, oral antidiabetic medications, and/or insulin. The exclusion criteria included: HbA1c > 10%, ESRD, active systemic or urinary infection, non-DKD, other glomerular diseases, autoimmune disease, neoplasia and mental disorders. The patient classification was conducted according to KDIGO Guidelines as follows: P1 – normal to mildly increased albuminuria; P2 – moderately increased albuminuria; P3 – severely increased albuminuria. All participants provided written informed consent.

The collection of blood was realised by venipuncture, stored in non-anticoagulant vacutainers, whereas the urine was collected in sterile vials. The samples were labeled with classified numerical codes and kept at at -80°C until sample asessment.

UHPLC-QTOF-ESI$^+$-MS assessment. A ThermoFisher Scientific UHPLC Ultimate 3000 instrument with a quaternary pump, Dionex delivery system, and MS detection equipment with MaXis Impact (Bruker Daltonics) was used to carry out the metabolomic profiling thorugh ultra-high performance liquid chromatography coupled with electrospray ionization- quadrouple time of flight mass spectrometry (UHPLC-QTOF-ESI$^+$-MS) technique.

Statistical analysis. The UHPLC-QTOF-ESI$^+$-MS analysis provided 420 serum and 550 in urine metabolites. Some of them were excluded according to low retention times, S/N values, m/z and peak intensities (PI). Ultimately resulted 136 molecules in serum and 196 in urine which were added to the Metaboanalyst 5.0 platform. The untargeted multivariate analysis was used to compare the serum and urine metabolites between the DKD group (P) and the control group (C). Fold Change, Volcano test, Pattern Hunter analysis, Partial Least Squares Discriminant Analysis (PLSDA), sparse PLSDA, and Variable Importance in the Pojection (VIP) were used to analyze metabolite discrimination between group P and group C. Additionally, the Random-Forest-based Prediction Test was used, and the P values were

determined using the Student's t-test. In the untargeted univariate study, the One Way Analysis of Variance (ANOVA), Fischer's Least Significant Difference (LSD), Pattern Hunter analysis, PLSDA, sparse PLSDA, Random Forest, and Biomarker analysis were utilized as analytical techniques.

6.3. RESULTS AND DISCUSSION

Phenylalanine and tyrosine

In this investigation, lower amounts of phenylalanine and tyrosine were found in the serum and urine of the DKD group compared to the C group. The normal to mildly increased albuminuria subgroup exhibits greater amounts of phenylalanine in both blood and urine, according to the individual comparison of the subgroups P1 vs. P2 and P1 vs. P3. Tyrosine also behaved in a manner similar to that of phenylalanine. It has been shown that low phenylalanine and tyrosine interact with diabetic nephropathy. In T2DM and in early stages of CKD both phenylalanine and tyrosine express abnormalities in their dynamic as it was pointed in this study. The phenylalanine pathway may be implicated in the early pathogenic process of DM development, and these changes may start years before the diagnosis of DM.

Metabolites of tryptophan

The multivariate analysis showed that the DKD group had higher levels of indoxyl sulfate compared to the control group. Additionally, the levels of indoxyl sulfate were found to be higher in urine than in serum. Univariate serum analysis has shown a progressive elevation in indoxyl sulfate levels that correlated with the progression of DKD (P1, P2, P3). Through a univariate analysis of urine, it has been observed that levels of indoxyl sulfate can distinguish the early stages of DKD, especially when comparing the normoalbuminuric group with other subgroups. Indoxyl sulfate may be a key player of early proximal tubular injury in DKD pathogenesis, even before albuminuria occurs. It may influence endothelial cell injury via increased formation of ROS. It also may be involved in BBB failure, a process driven by cytokines that trigger inflammation via OAT receptors.

In serum, serotonin sulfate expressed enhanced levels by the comparison of P1 with C and P2 subgroups, and decreased levels when comparing P1 with P3 subgroup. The urine analysis pointed to increased levels of this metabolite in P1 when compared to P2 and P3 patients and healthy controls. Serotonin sulfate may be implicated in the incipient renal fibrotic processes. This molecule implication, in cerebral and renal dysfunction, was not widely investigated and its trends are intriguing with regard to early DKD, thus deserving a special focus in future studies.

Retinoic acid signaling pathway

The findings of our study provide a comprehensive characterization of the retinoic acid signaling pathway, highlighting its potential significance as a contributing factor in the development and progression of DKD. The findings indicate a progressive increase in the concentration of ATRA in serum across the C, P1, P2, and P3 subgroups. Higher levels of ATRA were observed in the normoalbuminuric group when comparing P1 to C, P2, and P3 in urine.

In the normal to mildly albuminuria group, it may be observed an elevation in ATRA levels in urine and in serum. This group of patients also exhibits higher ATRA levels compared to the other groups (P2, P3 and C). The roles of ATRA in DKD may be as it follows: it may trigger the pancreas to generate insulin, enhances the differentiation of renal progenitor cells into podocytes, serves to halt the renal fibrotic process in the early stages of

DKD, may also trigger a compensatory mechanism to induce proliferation of endothelial cells and restore both endothelial integrity and neuronal recuperation.

Clinical relevance

These findings may be used in order to develop customized drug targets against metabolites derived from nitrogen metabolic pathway or retinoic acid signaling pathway. The use of probiotics, prebiotics and symbiotics, for redirecting the excretion of metabolic byproducts from the renal pathway to the fecal pathway, should also be considered.

6.4. CONCLUSION

In conclusion, this study identified metabolomic biomarkers associated with phenylalanine, tyrosine, tryptophan, and retinoic acid metabolism in patients with T2DM and normal to mildly increased albuminuria. These biomarkers indicate a distinct metabolic profile in early DKD patients, and could potentially indicate a shared kidney-brain pathogenic pathway. They may express a key role in podocyte, proximal tubule and renal and cerebrovascular endothelial dysfunction.

7. TARGETED METABOLOMIC INVESTIGATION OF GUT-DERIVED METABOLITES REVEAL A SPECIFIC PATTERN IN EARLY DKD IN T2DM PATIENTS

7.1. AIMS

This study is an extension of previous research that involved untargeted multivariate and univariate metabolomic analyses of serum and urine in patients with T2DM and DKD. The aim of this study is to provide a detailed analysis, by the quantification of the specific gut-derived metabolites that exhibited significant potential as biomarkers in the previous study.

7.2. MATERIALS AND METHODS

Patients' selection, sample collection, UHPLC-QTOF-ESI⁺-MS metabolite, statistical analysis and selection of metabolites.

The design of the second study follows the same methodology utilized in the first study. The second investigation involved the same group of patients and healthy controls. The study time frame, criteria for inclusion and exclusion of participants, the subdivision of groups, and the sample collection were consistent with the description provided in the chapter 6.2.

Reagents used for metabolite quantification

The reagents and chemical substances utilized for this investigation were as it follows: PLC-grade formic acid, HPLC/MS-grade formic acid, and acetonitrile. The pure standards used were: acetyl-L-carnitine hydrochloride, arginine, asymmetric dimethyl-L-arginine, hippuric acid, indoxyl sulfate potassium salt, sorbitol, p-cresyl sulfate, and creatinine. The instruments utilized in this study were a vortex mixer, Minicentrifuge Eppendorf.

The targeted analysis

The following stock solutions were used for the calibration solution and QC sample preparation: arginine, ADMA, L-acetylcarnitine, hippuric acid, sorbitol, indoxyl sulfate, and p-

cresyl sulfate and creatinine. These compounds were dissolved in methanol and/or ultra-pure water. To serve as an internal standard, a stock solution of DOXO was prepared, at a concentration of 2 mg/mL. The stock solutions underwent a sequential dilution process using a mixture of methanol and acetonitrile in a ratio of 2:1. This resulted in the generation of a series of operational solutions with varying concentrations, which were utilized for external calibration purposes. Simultaneously, 0.3 mL QC deproteinated samples were augmented with varying volumes of standard solutions. The validation process encompassed the assessment of linearity, specificity, precision, accuracy, as well as the determination of the limit of detection (LOD) and limit of quantification (LOQ). Two calibration curves were generated in this study (the external and internal standard curve).

7.3. RESULTS AND DISCUSSION

The levels of serum arginine decline, whereas ADMA increases progressively from C group to P1, P2 and P3 subgroups. Statistical analysis displayed variations in serum arginine levels when comparing the C group with the P subgroups, as well as between the P1 and P2 subgroups. ADMA levels proved significant differentiation between all the subgroups. In urine, there is a steady rise in the levels of arginine and ADMA, except for the P1 vs. P2 comparison where both metabolites display a reduction in their levels.

The observation that arginine is decreased and ADMA is increased in the P1 group, along with the observed variations between subgroups, suggests that these factors may play an underlying role in the development of early DKD. Specifically, they may contribute to (1) renal microvessel impaired vasodilatation (2) imbalances in the process of glycolysis and (3) ROS generation by enhanced oxidative stress.

Hippuric acid levels in serum were found to be decreased, from C subgroup to the P1-P3 subgroups. In urine, its levels were increasing following the same pattern. The statistical analysis of subgroups indicates that hippurate could potentially serve as an early biomarker for DKD when measured in serum. However, in urine, it does not differentiate between C group and P subgroups. The decrease in hippurate levels in serum from the C group to P1, P2 and P3 could be attributed to impaired mitochondrial activity in proximal tubular cells or due to low phenylalanine, which is a precursor of hippurate.

An increase in serum levels of indoxyl sulfate was observed as albuminuria progressed from normal to severe, with an analogous trend noticed in urine. Through further analysis, it has been found that indoxyl sulfate exhibits promising potential as a predictor of early DKD in serum. Increased indoxyl sulfate facilitates inflammation, vascular system calcification and microthrombi formation with subsequent damage of the glomerular filtration system. It may also cause proximal tubular cell apoptosis. Indoxyl sulfate has been identified as a potential biomarker for early-stage DKD based on preceding research and personal findings.

P-cresyl sulfate in urine displayed a consistent increase in its levels, which were found to be highly discriminatory when evaluating C vs. P1 vs. P2 vs. P3 and P1 vs. P2, by comparison. These differences were statistically significant, as indicated by the calculated p values. Our study indicates that p-cresyl sulfate contributes to the development of DKD. It is believed to cause similar endothelial cell and tubular epithelial cell damage as indoxyl sulfate, due to the presence of a pro-oxidative setting. Elevated levels of p-cresyl sulfate in the urine of our group with mildly to normal increased albuminuria, indicate that its discharge is not influenced entirely by the albumin loss and could lead to a clinical proximal tubular damage.

The results of this research displayed that serum levels of L-acetylcarnitine were highest by the comparison of P1 with C group and P2, P3 subgroups. The levels of this

compound, in urine, tend to gradually rise from the C group to P3 subgroup. Additional research determined that L-acetylcarnitine cannot be regarded as a biomarker for DKD due to its lack of statistical value resulted from the subgroup comparison.

Butenoylcarnitine is a type of carnitine with short-chain, that exhibits a serum level decline initially within the C group to P1 subgroup, followed by a spike in the P2 and P3 subgroups. The urine analysis indicates reduced concentrations in the C group and similar levels resulted by DKD subgroup comparison. Butenoylcarnitine elevated removal observed in our normal to mildly increased albuminuria subgroup, may indicate ongoing mitochondrial dysfunction in tubular epithelial cells.

Sorbitol levels, in the mildly to normal increased albuminuria subgroup, were found to be reduced when examined in relation to the C group, in the present research. Sorbitol levels tend to rise with the progression of albuminuria, particularly in individuals with severely increased albuminuria. It demonstrates a notable differentiation among C, P1, and P2. Thus, sorbitol has the ability to serve as a biomarker for early DKD by the aberrant activation of polyol pathway and abnormalities in NADPH and NAD^+ which are involved in NO production.

7.4. CONCLUSION

This second study highlights arginine, dimethylarginine, hippuric acid, indoxyl sulfate, butenoylcarnitine, and sorbitol, in serum, and p-cresyl sulfate in urine as potential biomarkers associated with early metabolic disruptions in DKD. The quantitation of metabolites derived from gut microbiota offers new insights into the metabolism of carnitines, amino acids and uremic toxins. These findings could be beneficial to standardize and incorporate the metabolites found in customized metabolic assessment panels for early diagnosis of DKD.

8. GUT MICROBIOTA DERIVED BIOMARKERS ARE LINKED TO THE DYSFUNCTION OF RENAL AND CEREBROVASCULAR ENDOTHELIUM AND WITH PODOCYTOPATHY AND PROXIMAL TUBULOPATHY IN EARLY DKD

8.1. BACKGROUND AND AIMS

The eye, kidney and the peripheral and central nervous systems are most frequently affected by complications of T2DM. This study is focused on the role of specific metabolites (arginine, hippuric acid, indoxyl sulfate, p-cresyl sulfate, L-acetylcarnitine, butenoylcarnitine, and sorbitol) within podocytes, tubules, and renal and cerebral endothelial cells. This investigation aims to assess the potential relationship between the mentioned metabolites and early cerebral vessel remodeling and renal damage in patients suffering from T2DM.

8.2. Materials and methods

The participant selection and sample preparation were the same as the ones described in the first study. In addition, in this study there were performed additional assessment methods such as ELISA and cerebrovascular Doppler Ultrasound. ELISA technique was used to determine MCP-1, ICAM-1, KIM-1, NAG, PDX, and STP. By cerebrovascular Doppler ultrasound, there were evaluated the right common carotid arteries (R-CCA) and the right middle carotid arteries (R-MCA). This kind of assessment provided the following parameters: intima-media thickness (IMT), pulsatility (Pi) and resistance indices (Ri). In addition, by Doppler Ultrasound was assessed the cerebrovascular reactivity

(CVR), which reflects the autoregulatory vasodilation of cerebral blood vessels upon exposure to a vasodilator stimulus, such as hypercapnia. CVR was evaluated by the determination of breath holding index (BHI).

8.3. Results and discussion

In this study, serum arginine displays an inverse correlation with both IMT and ICAM-1 in a predictive pattern. Decreased levels of arginine may suggest endothelial dysfunction due to decreased renal synthesis. The correlation between IMT and arginine might indicate the presence of endothelial dysfunction in the common carotid arteries. The correlation between arginine and ICAM-1 implies a simultaneous dysfunction of renal endothelial cells. Therefore, serum arginine may serve as a reliable biomarker for early detection of cerebral atherosclerosis and renal endothelial dysfunction in patients suffering from DKD with associated normal to mildly increased albuminuria.

A positive correlation was observed between serum butenoylcarnitine levels and both IMT and MCP-1. These results are further supported by the presence of statistically significant p values. Therefore, this study provides evidence to substantiate the claim that butenoylcarnitine contributes to the development of renal and cerebrovascular endothelial dysfunction. The inclusion of this key metabolite in biological panels has the potential to address the issue of early DKD.

It was observed a positive association between indoxyl sulfate and indicators of endothelial dysfunction, such as ICAM-1 and Pi R-ACI. Furthermore, we observed a clear distinction between the P1 subgroup compared to both the C and P2 subgroups. The correlation between this byproduct and Ri R-ACI suggests its involvement in the process of cerebral vessel remodeling, which is associated with early cerebrovascular endothelial cell damage in the initial stages of DKD. The link between indoxyl sulfate and ICAM-1 indicates the potential involvement of this compound in the processes underlying systemic endothelial dysfunction. Additionally, it suggests that indoxyl sulfate may contribute to a selective damage on renal tubular cells by promoting the expression of ICAM-1 at this specific level.

The findings of our research indicate a negative association between serum sorbitol and BHI, as well as a positive association with ICAM-1, when comparing the P1 subgroup to both the C and the P2 subgroup. CSVD is a pathological condition that includes impaired endothelial function, which may be identified through the utilization of MRI or cerebral Doppler ultrasound. These methods may reveal cerebral lacunes, microinfarcts, microbleeds, and white matter hyperintensities, as observed in MRI, or they may suggest impaired vasodilation using advanced neurosonological techniques. Due to the limitations of current diagnostic techniques in identifying early endothelial damage, the measurement of sorbitol levels could potentially be valuable to analyze BBB performance and CVR. Consequently, sorbitol determination holds promise as a prospective therapeutic target.

In the present investigation, the inclusion of urine L-acetylcarnitine as a DKD biomarker may not be warranted as it did not reach statistical significance. The presence of butenoylcarnitine in the urine exhibited a significant correlation with uACR and PDX levels. This finding suggests that buteonylcarnitine has the potential to serve as a biomarker for assessing podocyte injury in DKD.

The urinary indoxyl sulfate levels exhibit a positive correlation with PDX and uACR. This observation not only indicates the presence of oxidative stress in endothelial cells but also suggests damage occurring at the podocyte level. Urinary indoxyl sulfate may be regarded as a biomarker for assessing podocyte damage in DKD. However, it may not be effective in distinguishing the P1 subgroup from the other subgroups.

The results of the multivariable analysis indicated a positive correlation between urinary p-cresyl sulfate and both KIM-1 and uACR. The findings of our study suggest that urinary p-cresyl sulfate may serve as a biomarker for proximal tubular damage in diabetic patients who are in the early stages of DKD. Moreover, the early detection of DKD may be facilitated through the measurement of urinary p-cresyl sulfate providing to the clinicians the opportunity to rapidly initiate specific therapeutic interventions.

8.4. Conclusion

In summary, this last research underscores the observation that individuals afflicted with early DKD exhibit a distinct profile of metabolites, which may originate from the gut microbiota. Therefore, it can be concluded that serum arginine, serum indoxyl sulfate and serum butenoylcarnitine serve as biomarkers linked to renal endothelial dysfunction and to early common carotid artery atherosclerosis. Additionally, sorbitol in serum is a biomarker that indicates BBB damage, which is closely associated with CSVD development. Simultaneously, the analysis of urine indicates that urinary butenoylcarnitine serves as an indicator of podocyte injury, alongside urinary indoxyl sulfate in DKD. Urinary p-cresyl sulfate was identified to be a biomarker of proximal tubular damage during the initial phases of DKD