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PhD THESIS

IDENTIFICATION AND CHARACTERIZATION OF GLYCOSPHINGOLIPIDS AS URINARY BIOMARKERS IN DIABETIC KIDNEY DISEASE. A STUDY BASED ON HIGH-RESOLUTION MASS SPECTROMETRY COUPLED WITH NANOELECTROSPRAY TECHNIQUE

A B S T R A C T

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INTRODUCTION

Early diagnosis of diabetic kidney disease (DKD) is extremely important, as it might enable prompt intervention, thereby preventing the advancement of this condition, and the possibility of developing end-stage renal disease (ESRD). Therefore, an in-depth assessment of renal function and the screening of markers for renal dysfunction, such as urine albumin-to-creatinine ratio, serum creatinine level, and estimated glomerular filtration rate tests, are currently considered necessary for early detection of DKD. Given the fact that even early stages of DKD may increase the risk of cardiovascular morbidity and mortality, it is imperative to improve our knowledge regarding the pathogenesis of this microvascular complication, and to develop therapeutic interventions.

It is therefore conceivable that the lack of available sensitive diagnostic biomarkers is the most significant limitation in the way of an early DKD diagnosis. Over the years, many research groups have investigated circulating, urinary and renal tissue biomarkers for DKD early diagnosis and progression, based on the pathogenesis of this microvascular complication.

Glycosphingolipids (GSLs) are a type of glycolipids, that feature a hydrophobic lipid component (a sphingoid base or a ceramide), which is linked to at least one oligosaccharide residue.

Gangliosides (GGs) are receiving substantial interest among all GSLs. Currently, the accurate structural investigation of GGs is not considered an issue in determining and describing the intricate arrangement of all species, not solely of those, that are abundantly expressed. This feature is attributed to the outstanding advantages offered by mass spectrometry (MS), which serves as a fundamental analytical instrument for the examination of this molecules.

The aims of the present study were to further expand prior research attempts, that have provided insights into subjects related to glomerular and tubular damage in patients with type 2 diabetes mellitus (type 2 DM). The current investigations seek to offer novel perspectives by identifying potential early biomarkers of the diabetic kidney disease, which could facilitate the development of innovative therapeutic interventions, that focus on preventing or decreasing the disease progression.

The objectives of the experimental part of the thesis refer to:

- *The first study* focused on the optimization and validation of nanoelectrospray ionization high-resolution mass spectrometry (nanoESI HR MS) method on an Orbitrap instrument, using brain hemangioma samples. The objective was to validate various sample preparation assays, including extraction, separation, purification, identification, and quantification of gangliosides, and to establish the optimal laboratory conditions for conducting this procedures on complex biological matrices, in order to detect biological structures with diagnostic and prognostic role.
- *The second study* aimed to identify and characterize urine gangliosides with potential role as diagnostic bioindicators in the early DKD. For this purpose, we used the previous validated bioanalytical platform, based on nanoESI HR MS on an Orbitrap instrument operating in negative ion mode for both MS screening and tandem MS (MS/MS) fragmentation, for the measurement, profiling, and detailed structural analysis of urinary gangliosides in type 2 DM patients.

- *The third study* reflects a critical assessment of state of the art in ganglioside analysis in various body fluids, by contemporary liquid chromatography techniques, thin-layer chromatography, hydrophilic interaction liquid chromatography, and ion mobility separation hyphenated with mass spectrometry.

Keywords: diabetic kidney disease; brain hemangioma; ganglioside biomarkers; glycomics; high-resolution tandem mass spectrometry; separation techniques

REVIEW OF THE LITERATURE

The first part of this thesis is composed of *three chapters*, and contains literature informations regarding the defintion and pathogenesis of chronic kidney disease (CKD), diabetic kindney disease (DKD), the state of the art in biomarkers discovery of DKD, as well as concerning the role of gangliosides in several pathologies, such as kidney diseases, brain tumors, and neurodegenerative pathologies.

1. CHRONIC KIDNEY DISEASE AND DIABETIC KIDNEY DISEASE. DEFINITION, TERMINOLOGY AND CONCEPT

Chronic kidney disease is a long-term, progressive condition characterized by structural and functional kidney abnormalities resulting from a variety causes. It is important to detect and address the underlying cause of chronic kidney disease, in order to reduce the risk of further complications and to delay the progression of this condition. Diabetes, hypertension, and glomerulonephritis are among the most common aethiologie of chronic kidney disease.

Type 2 DM represents one of the world's leading health concerns. The effects of type 2 DM comprise microvascular complications and approximately 40% of patients will develop diabetic kidney disease. Growing evidence suggests that diabetes is the leading cause of chronic kidney disease and end-stage renal disease. Since even early stages of **diabetic kidney disease** could pave the way to a high risk of cardiovascular mortality and morbidity, it is thought to be crucial to gain a better understanding of the pathogenesis of this microvascular complication and to approach therapeutical strategies.

Albuminuria has conventionally been recognized as the initial clinical manifestation of diabetic kidney disease, serving as a biochemical marker for DKD progression. Under certain situations, the regression of albuminuria may occur, either spontaneously or as a result of renoprotective treatment. Moreover, Albuminuria has been recognized as an independent risk factor for cardiovascular disease, linked to a greater occurrence of cardiovascular morbidity and mortality. Still, further studies have shown that a high percentage of type 2 DM patients could remain normolbuminuric, although serum creatinine arises. Hence, growing data suggests that the conventional view of DKD, in which the decline in GFR is preceded by high values of albuminuria, is not necessarily accurate. The focus has switched to the proximal tubule (PT), which may have a crucial role in the early pathophysiology of this renal microvascular complication. Highlighting the role of the PT in determining albuminuria, has diminished the relevance of glomerular barrier abnormalities.

2. STATE OF THE ART IN BIOMARKERS DISCOVERY OF DIABETIC KIDNEY DISEASE

In recent years, notable progress in comprehending the pathogenesis of diabetic kidney disease has been realized. There exists a necessity to update the present interest of the pathological alterations, the disease progression and the treatment of patients with

diabetic kidney disease. Renal damage in diabetes is not only in the glomerular compartment, but also in the tubulo-interstitial and vascular compartment.

New biomarkers are expected to intervene in order to reduce disease prevalence and to improve the prediction of DKD onset and progression.

This section describes the innovative biomarkers, that represent one biochemical pathway of the disease. Some of these biomarkers have been shown to predict the risk of developing DKD. The biomarkers that are known to evaluate renal impairment in type 2 DM patients can emerge from different components of the nephron: podocyte damage biomarkers – nephrin, podocalyxin; glomerular basement membrane level – collagen, laminin; podocyte/endothelial biomarkers – vascular endothelial growth factor (VEGF); proximal tubule damage biomarkers – neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), N-acetyl-β-D-glucosaminidase (NAG), angiotensinogen.

During the last few years, the interest has shifted to the use of omic approaches, such as proteomics, glycomics, metabolomics, and transcriptomics, as they become a resourceful strategy in biomarker identification.

3. GANGLIOSIDES AS BIOMARKERS

Gangliosides belong to the class of glycosphingolipids, and comprise a hydrophobic ceramide moiety and a hydrophilic oligosaccharide chain. The hydrophobic component consists of a sphingoid base and a fatty acyl chain, both varying in structure between species. Additionally, the length and composition of the hydrophilic part show variability. GGs possess distinctive characteristics due to the existence of one or more sialic acid units in their oligosaccharide chains. They constitute key components of the plasma membrane, where they connect to proteins, phospholipids, and cholesterol to form caveolae microdomains (lipid rafts).

Gangliosides as biomarkers of kidney diseases

Recently, polar glycosphingolipids have been studied, due their potential role as biomarkers in early diagnosis of a number of different kidney disorders of genetic or non-genetic origin, such as Fabry disease, idiopathic and secondary glomerulonephritis, polycystic kidney disease, and renal cancer.

Ganglioside mapping in human brain. Biomarkers of brain diseases

Considering the fact that the brain is not easy to study, and that the pathological processes have already taken place before the occurence of clinical signs, the comprehension of various neurological disorders has advanced significantly in recent years. A detailed mapping and structural characterization of acidic glycosphingolipids is essential in order to establish the role of these components in healthy and diseased central nervous system. Severe neurodegenerative diseases can be caused by either the absence or the excess of GGs. Mutations of ganglioside synthesis are assumed to be also the cause of congenital epileptic syndromes. Aberrant abundance might also emerge in some neurodegenerative diseases (Huntington's disease, Alzheimer's disease, Parkinson's disease), in maturation and aging, as well as in benign vascular brain tumors and malignant tumors.

PERSONAL CONTRIBUTIONS

Currently, MS is regarded as one of the most effective, precise, and sensitive techniques for the analysis of GGs. MS can be utilized to obtain valuable compositional and structural information for biochemical markers detection in complex samples. In such cases, diverse separation methodologies, such as electrophoresis or chromatography, are commonly employed prior to MS assessment. This part of the paper contains three clinical studies.

1. OPTIMIZATION OF HIGH-RESOLUTION MASS SPECTROMETRY FOR GANGLIOSIDE ANALYSIS

The present investigation has focused on the optimization and validation of nanoESI HR MS utilizing the Orbitrap instrument in the negative ion mode. These methods were subsequently utilized for the purpose of mapping and conducting structural assessment of GG mixtures obtained from human cavernous hemangioma of a 42-year-old patient. Hence, novel techniques in glycomics related to aberrant tissue gangliosides, were implemented. The objective behind the improvement of this analytical method was to evaluate different classes of molecules, in order to establish the optimal conditions for conducting this procedure, and to validate various sample preparation assays, including extraction, separation, purification, identification, and quantification of ganglioside classes and species.

The experiments involving MS were carried out using a LTQ Orbitrap Velos Pro™ mass spectrometer, which was equipped with an offline nanoES source.

After conducting a comprehensive analysis of the spectrum and performing precise mass calculations, the 24 identified ions were attributed to distinct ganglioside species. The obtained mass spectra exhibit a very complex molecular ion pattern, encompassing various species, that are distinguished by a diverse range of oligosaccharide chains and ceramide part compositions, along with several biologically significant changes, including *O*-acetylation and *O*-fucosylation. One modified *O*-Ac-GT1 and one modified *O*-Ac-GM4 gangliosides were detected.

Therefore, the utilization of HR MS has the potential to identify numerous previously unknown species. Moreover, the current results reveal new insights, such as the identification of gangliosides with reduced sialylation and shorter glycan chains, that are more prevalent, and the detection of asialo species GA1 (d18:1/18:0) in brain hemangioma. The presence of O-Ac-GM4 (d18:1/16:0) species and the prevalence of GD2-type components in HE42 align with the findings of a prior investigation, which associates these structures with decreased malignancy levels in brain hemangioma tumors.

Moreover, the HR MS analysis revealed that the HE42 ganglioside mixture consisted mainly of species featuring shorter glycan chains and a decreased number of sialic acids.

For a closer inspection of these species which dominate hemangioma gangliosidome, HE42 native mixture was subjected to separation by high performance thin layer chromatography (HPTLC). The separation by HPTLC and individual analysis of GD and GT fractions enhanced the detection of minor components, which, because of the high heterogeneity of HE42 could not have been distinguished by the MS screening of the native mixture as a whole.

These results not only show the complexity of the gangliosidome associated to aberrant human matrices, but also that in the current study we succeeded a proper opitmization of the nanoESI HR MS conditions for detection and identification of even minor species of biomarker value in highly complex mixtures from crude human extracts. The species GD3(d18:1/16:0), GD2 (d18:0/13:0), GD3(d18:1/26:1) and *O*-Ac-GT1(d18:0/22:0) seem to be of major interest for the early diagnosis of carvenous hemangioma.

2. THE ASSESSMENT OF GANGLIOSIDES IN EARLY DIABETIC KIDNEY DISEASE OF TYPE 2 DIABETES MELLITUS PATIENTS USING HIGH-RESOLUTION TANDEM MASS SPECTROMETRY

Given the paucity of data available so far in the field of glycolipidomics concerning patients with diabetic kidney disease, the present study aimed to address the problematics of detection and characterization of gangliosides in the urine of type 2 DM patients with DKD.

For this purpose, after the optimization of high-resolution (HR) mass spectrometry (MS) and tandem MS (MS/MS) methods, by applying the previous determined parameters, we continued our research by implementing these techniques to precisely identify the presence of GGs in the urine of DKD patients.

In a cross-sectional pilot study, type 2 DM patients and control subjects were evaluated with an aim to optimize and verify the protocol. We compared the gangliosidome of 30 type 2 DM patients, assigned into 3 categories, based on the UACR: 10 normo- (UACR < 30 mg/g; named A1), 10 micro- (UACR 30–300 mg/g; named A2), and 10 macroalbuminuric patients (UACR > 300 mg/g; A3), to that of 10 healthy control subjects (C). LTQ Orbitrap Velos Pro consists of a linear ion trap and an Orbitrap mass analyzer, with excellent mass resolution and reliability together with an effective fragmentation technique, collision-induced dissociation (CID), in order to obtain a thorough analysis of complex urine samples. All mass spectra were acquired under identical conditions, in negative ion mode.

The number and type of GG components revealed in the A1, A3, and C samples differed significantly, whereas there was no difference between A1 and A2 samples. The performant MS platform enabled the detection and recognition of 37 different urine gangliside and fucoganglioside species in the three samples. Upon conducting a thorough analysis of the structures, it was observed that the A3-macroalbuminuric study group exhibited the greatest number of unique ganglioside compounds, i.e.,19, among all samples. Following A3, sample C contained 12 different GG species, while sample A1 comprised 10. The detected species correspond to 15 distinct classes, involving changes to the main glycan core. In contrast to the A1 and C samples, A3 exhibits a higher number of species with longest O-glycan chain associated to the G1 class, and a greater variety of ceramide forms. Additionally, A3 displays a superior number of expressed GG classes, i.e., 12, compared to only 6 in the A1, and 6 in the C samples. Furthermore, A3 contains a greater number of GG, that display biologically relevant modifications such as non-carbohydrate O-acetyl, carbohydrate O-fucosyl, and O-GalNAc. Notably, A3 is the sole group that contains GG structures modified by O-GalNAc attachment, particularly GalNAc-GS1(t18:1/18:0) molecule, identified at m/z 1176.8266, and GalNAc-GQ1(d18:1/18:0) molecule, detected at m/z 1310.1271.

Furthermore, given the observation that 7 trisialo GT1, 4 pentasialo GQ1, and 1 heptasialylated GS1 were discovered, the high sialylation degree could serve as another

particular characteristic of the macroalbuminuric group. In addition to di-, tri-, and tetrasialylated GGs in A1 samples, the ion detected at m/z 676.5569 was ascribed, according to mass calculation, to the pentasialo gangliotetraose GP1(d18:1/18:0). Such evidence regarding the sialylation status of the examined samples demonstrates the precocity of these alterations, even in the normoalbuminuric stage. Clearly, the level of sialylation rises as the condition worsens, making it a molecular marker to be regarded not just for the early DKD diagnosis, but additionally for an in-depth assessment of the disease progression and therapeutic efficacy.

Upon conducting a thorough analysis of the aglycone component, it has been observed that the gangliosides present in the studied samples display also variations in the composition of the lipid part, in addition to the notable differences in the structure of the glycan chain. From the data, two species containing trihydroxylated sphingoid bases of the fatty acid were detected in both A1 and A3 samples. The normoalbuminuric group was found to contain GT1(t18:0/18:0) and GT1(t18:0/20:0), while the macroalbuminuric group exhibited complicated structures, such as trihydroxylated GT1(t18:1/24:3) and Fuc-GT3(t18:1/18:3).

Using MS/MS by HCD, a few ions were found to be predictive for certain isomers associated with the positioning of Neu5Ac within the glycan chain. The presence of GQ1d(d18:1/18:0), GT1α(d18:1/18:0), and GT1b(d18:1/18:0) isomers in the macroalbuminuric group has been discovered to be associated with DKD progression, based on the informative sequence ions obtained from the structural analysis.

The majority of research studies, that aim to characterize the renal gangliosidome, mainly employ experimental models of DN. To the best of our knowledge, this study represents the starting point for human translational investigation, and documents a particular urinary ganglioside profile in individuals diagnosed with Type 2 DM. Our study highlights the correlation between the sialylation level of urinary GGs, and the composition of their ceramides, with early DKD diagnosis.

3. CRITICAL ASSESSMENT OF STATE OF THE ART IN GANGLIOSIDE ANALYSIS BY LIQUID-PHASE SEPARATION TECHNIQUES HYPHENATED TO MASS SPECTROMETRY

As shown in our previous two studies, mass spectrometry is widely regarded as a highly effective tool for gaining comprehensive understanding of the complex glycan structures. MS, along with tandem MS (MS/MS), have the capability to analyze glycan compounds. However, they are insufficient for a thorough analysis of isomeric glycan structures. Therefore, multistage MS (MSn) has the capacity to conduct in-depth analysis on these structures. In the case of complex biological specimens (urine, blood, milk, CSF), it is crucial to first separate glycan structures, due to the necessity of isolating individual structures from a vast glycan mixture. The process of separation becomes more important in cases where glycan structures appear in small amounts and require isolation from other structures that are more abundant. Additionally, the process of separation contributes to improved ionization performance, by avoiding ion suppression. Therefore, the hyphenation of MS with liquid-phase separation techniques, is widely adopted.

Unlike in SNC, GGs are present in body fluids in rather small quantities. Their composition varies in accordance with various factors, such as pathological conditions, and may have diagnostic relevance.

Hence, the third study reviews the significant accomplishments of modern separation techniques coupled with mass spectrometry, in the study of GGs present in body fluids.

Modern approaches, based on liquid-phase separation techniques coupled to mass spectrometry, and characterized by ultrahigh sensitivity, separation performance, analysis rapidity, and accuracy of identification, are highly efficient and powerful for detecting the presence of GGs and isoforms in biological fluids. However, the clinical use of these approaches, in order to identify gangliosides as biochemical markers, is still limited.