Spatial glycomics and kidney disease

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Abstract

Glycans are critical for the kidney's physiological and pathological cellular functions, and our ability to reveal their spatial distributions within tissues has helped us reveal how these carbohydrate moieties are involved in many of these processes. This review discusses the role of different types of glycans in kidney biology and disease, common approaches used for glycan imaging, and how glycan imaging has helped us better understand kidney pathology. We mainly focus on emerging methods using mass spectrometry imaging (MSI) because this technology is untargeted and provides complete information on glycan composition compared to the other methods, such as lectin and metabolite labeling, which are targeted and often inform only on the specific part of a glycan structure. We especially focus on protein *N*-glycosylation, as this is one of the most common post-translational modifications, and these moieties play a vital role in renal structure and function. The recent advancements in MSI of *N*-glycans we reviewed have provided new insights into the pathophysiology of the kidney and paved the way for clinical application.

Keywords

Glycans, *N*-glycosylation, mass spectrometry imaging, lectin staining, metabolite labeling

Glycans in the physiology and pathology of kidney

The study of kidney glycans is essential, as these carbohydrate polymers play critical roles as anchoring sites for cell adhesion, extracellular matrix molecules, signaling receptors, immune cells, lectins, and pathogens[1]. Moreover, glycogen, a highly branched glucose storage polymer, which is naturally present in low abundance in the kidney, can accumulate in diabetic kidneys[2]. The glycans on newly synthesized glycoproteins also contribute significantly to essential processes such as protein folding, intracellular transport, and secretion.[3] Furthermore, glycan aberrations are central to numerous biological events, including disease progression.[3, 4] Research on kidney glycans holds immense potential for advancing our understanding of these processes, their implications in renal disease, and in defining disease states.[1, 3]

Glycans comprise various monosaccharide units, organized in carbohydrate moieties of different composition, lengths, and branching patterns, free or covalently attached to other biomolecules such as lipids and proteins (**Figure 1**)[4]. Glycans modify proteins either through controlled enzyme-mediated processes (glycosylation), where oligosaccharides are covalently attached and modulate protein function, or through non-enzymatic random chemical (Maillard) reactions (glycation), where monosaccharides are covalently attached and alter the structure and function of proteins[5].

Glycation end products (AGEs) can accumulate in the kidneys of patients with chronic kidney disease (CKD) due to increased production and decreased clearance[6]. This accumulation can cause kidney damage by cross-linking extracellular matrix (ECM) proteins, leading to stiffness, glomerulosclerosis, atherosclerosis, and thickening of the

glomerular basement membrane (GBM)[6]. Both *O*- and *N*-glycosylation are important for normal kidney function. Protein *O*-glycosylation occurs on the *Ser* or *Thr* residue, whereas *N*-glycosylation occurs on the *Asn* residue. *O*-Mannosyl glycans regulate glomerular filtration by connecting podocyte foot processes to the GBM,[7] and abnormal *O*-glycopeptides can contribute to immune-mediated kidney diseases, such as IgA nephropathy (IgAN).[8]

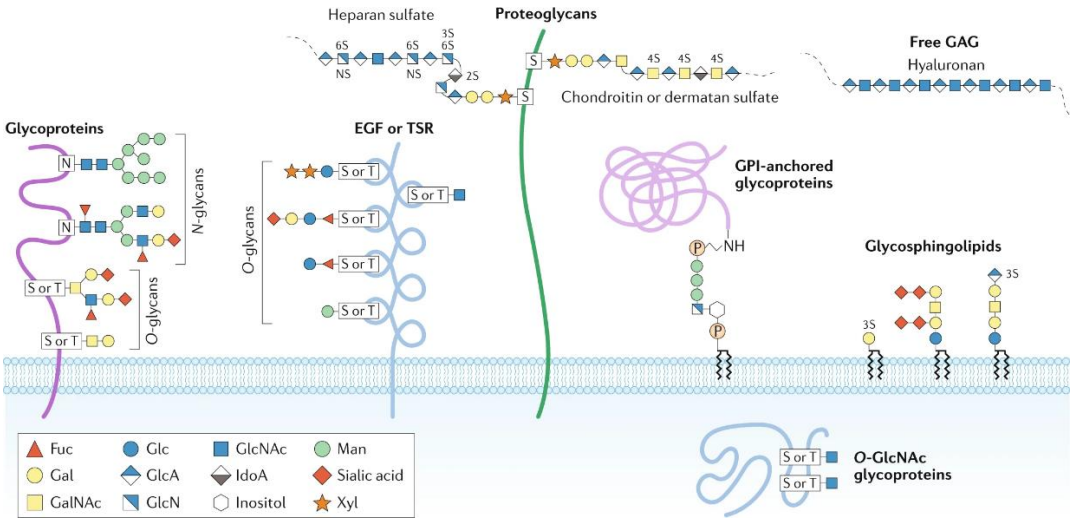

Figure 1. Diversity of glycans in humans. Adapted from ref[4]. Fuc: fucose, Gal: galactose, GalNAc: N-Acetyl-galactosamine, Glc: Glucose, GlcA: Glucuronic acid, GlcN: Glucosamine, GlcNAc: N-Acetyl-galactosamine, IdoA: Iduronic acid, Man: mannose, Xyl: xylose.

Protein *N*-glycosylation is of notable importance, as *N*-glycans are the major class of carbohydrate modifications present in 90% of all glycoproteins.[9] Molecularly, *N*-glycans consist of a highly diverse set of structural classes that contain a number of different monosaccharide compositions and branch structures (**Figure 1**).[10] Changes in

glycoprotein function due to variations in the linkage type, monosaccharide units, and branching alter cellular phenotypes are known to be involved in numerous disease states.[3] Abnormal *N*-glycosylation participates in the pathogenesis of kidney disease through various mechanisms, such as influencing protein maturity, exposing protein immunogenicity, and promoting inflammatory response.[11]

Recent studies have shown a substantial link between *N*-glycosylation signatures and the progression of many renal diseases,[12] including diabetic nephropathy, polycystic kidney disease, and renal cell carcinoma.[5, 13-15] For example, higher HbA1c in type 1 diabetes is associated with changes in the serum *N*-glycome.[5] These changes have been shown to regulate the epidermal growth factor receptor and transforming growth factor (TGF)-β pathways implicated in diabetic kidney disease (DKD).[16] Other studies showed that glycosylation patterns of kidney proteins differ in diabetic nephropathy and renal tumor development.[13] Proper *N*-glycosylation is vital for the correct membrane localization of several essential component proteins (e.g., nephrin, podocin, and Crumbs2), enables their interactions with other molecules, and further sustains the glomerular filtration barrier (GFB) function.[11] Furthermore, studies indicate that the loss of negatively charged sialic acid residues on glycoproteins in podocytes is responsible for decreased filtration of the glomerular capillaries.[17]

Despite the noted huge impact of glycans on renal physiology and pathology, we know very little about how particular glycome chemistry affects the phenotype, cellular function, and pathology of kidney cells because most of our knowledge is confined to measuring glycome at the serum or whole tissue scales. These bulk-based methods cannot

differentiate between cells, cell types, or functional tissue units (i.e., microanatomical regions). As such, imaging glycans to resolve their chemistry at the cellular and functional tissue unit resolution in human tissues is essential for understanding these molecules' physiological and pathological cellular functions. Approaches for spatial glycomics discussed in this review have helped localize aberrant glycosylation and better understand the chemistry of glycans involved in kidney function and disease.

Approaches for glycan imaging

Historically, methods based on recognition with probe-bearing lectins or antibodies were commonly used for glycosylation analysis in kidney disease (**Figure 2**).[5, 18-21] Lectins can recognize structures as varied as the monosaccharides sialic acid and fucose to higher-order structures such as the conserved core region of *N*-glycans. However, lectins typically have low affinity for their glycan epitope and require multivalency for high-avidity binding. Moreover, because lectins are specific for epitopes and not entire glycan structures, these approaches could not sufficiently characterize glycan molecules, limiting their use in biomarker discovery and mechanistic studies.[22]

Alternatively, glycans can be imaged by metabolic labeling with chemical reporters and subsequent ligation to fluorescent probes. This technique has enabled the visualization of glycans in living cells and even in live organisms.[23, 24] Here, the chemically reactive moiety is incorporated into target glycans by metabolic labeling with a non-endogenous monosaccharide substrate, where the chemically reactive group is termed a "chemical reporter." This moiety is small enough to be ignored by the cell's metabolic enzymes and inert to the endogenous chemical functionality of the cell (criterium termed

as “biorthogonality”). In the second step, the reporter group is visualized by a covalent reaction with an imaging probe. Most glycan subtypes, except glycosaminoglycans, have been imaged by metabolic labeling with azido or alkynyl monosaccharides. Nonetheless, this approach is also limited to differentiating glycan subclasses but not individual glycans within the subclass.[23]

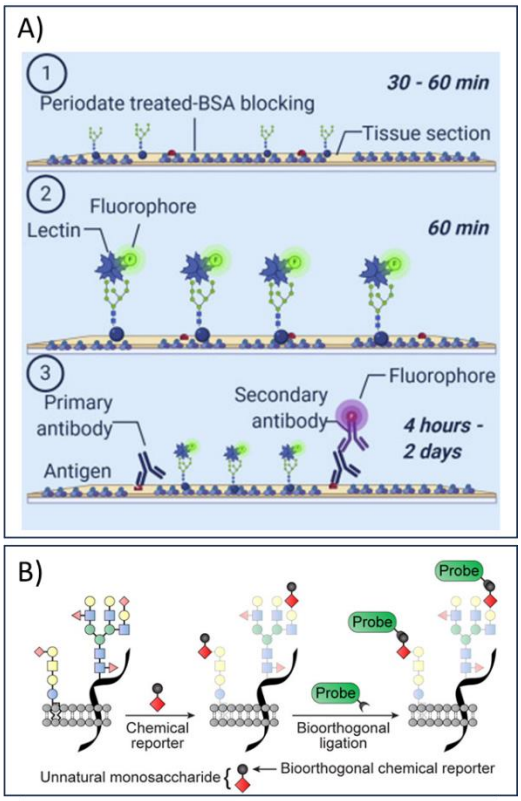

Figure 2. Glycan imaging through A) combined fluorescent lectin/immunohistochemistry and B) bioorthogonal chemical reporters. Adapted from ref[23] and ref[21].

Currently, mass spectrometry (MS) is a prime technique for glycosylation analysis due to its high sensitivity, high-duty cycle, and ability to provide structural information of

the glycans. All of these measurement attributes are needed to meet the clinical requirements of glycosylation profiling in large cohort studies.[11] More recently, the development of matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) approaches for mapping the distributions of *N*-glycans across biological specimens has shown tremendous potential, in part because it can provide insights into the tissue heterogeneity of *N*-glycosylation. [25]

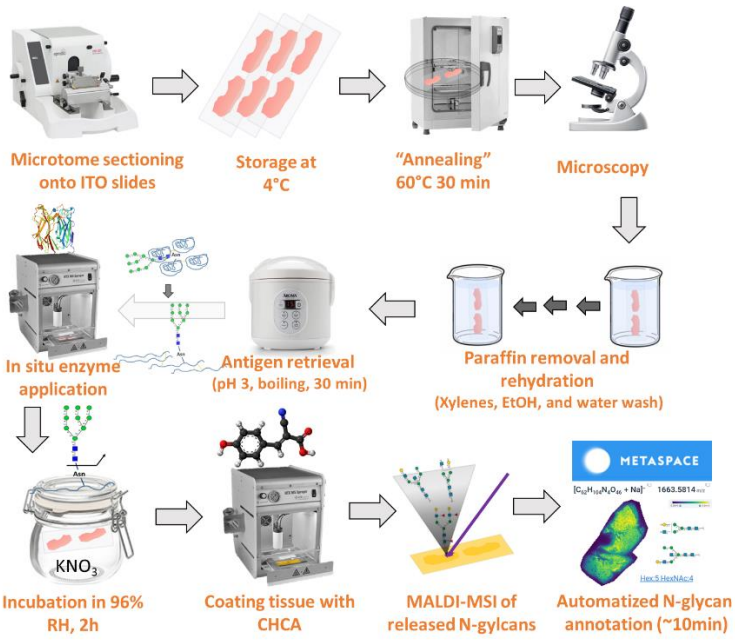

Figure 3. General workflow for *N*-glycan MALDI-MS imaging.

This method, originally developed by Dr. Richard Drake, relies on the enzyme peptide: *N*-glycosidase (PNGase F) to be sprayed over the tissue section, which will *in situ* release the *N*-glycans from carrier glycoproteins (**Figure 3)**.[26] The protocol starts with sectioning of formalin-fixed paraffin-embedded (FFPE) tissue blocks (or fresh frozen tissues), section mounting on a glass substrate (often conductive for MALDI-MSI),

dewaxing the tissue sections (per FFPE tissues), exposing the glycans from glycoproteins (antigen retrieval) in boiling acidic buffer, and *in situ* enzyme digestion within a temperature and humidity controlled environment. Next, the application of the MALDI matrix is performed on the sections. Typically, α-Cyano-4-hydroxycinnamic acid (CHCA) is used as this matrix, which helps in the ionization of *N*-glycans. Finally, samples are analyzed with MALDI-MSI, where a UV laser is used to locally ablate, ionize, and introduce *N*-glycans into the gas phase, which can be directed into a mass spectrometer for measuring their relative abundance across the ablated areas. Those ablated areas are usually ≥25 μm μm in diameter and are usually separated by dozens of microns or less, often approaching cellular resolution in human tissues.[25]

Compared to lectin and other glycan-binding tissue staining protocols that can only partially characterize glycan composition (e.g., specific epitope), MALDI-MSI is a broad and untargeted method that measures the exact mass of the detected *N*-glycans. The detected masses can then be used with specialized databases[27] to reveal *N*-glycans' composition (e.g., type and number of monosaccharides) in a particular tissue area. This makes this approach ideal for biomarker discovery and mechanistic studies.

Even though this technique is suitable for analyzing fresh-frozen tissue samples, it shows its full potential in analyzing FFPE tissue blocks, where sensitivity is much higher compared to fresh-frozen sections.[28]. As such, compared to MALDI-MSI-based spatial metabolomics, lipidomics, and proteomics analysis, which require fresh-frozen and non-fixed samples, spatial glycomics offers the advantage of analyzing archived blocks and

samples where morphology and cell structures are better preserved. This consequently results in the ability to more accurately gain insight into the cellular origin of *N*-glycans.

One critical step in the enzyme-assisted MALDI-MSI workflow is during the *in situ* digestion step, where the balance of water content on the sample surface is important. Specifically, there should be enough water for efficient hydrolysis, but not enough to cause delocalization of released molecules, which can be caused by accumulated condensation on the sample. Careful optimization of the humidity conditions in the enzymatic digestion sample preparation step can achieve sensitive and accurate spatial information on *N*-glycan distribution. We designed a digestion chamber that uses saturated salt solutions to maintain constant relative humidity (RH). It was shown that the best performance for enzymatic-assisted MALDI-MS imaging analysis of *N*-glycans from human kidney biopsies was achieved using a saturated solution of KNO_3 that maintains an 89% RH.[29] Under these conditions, maximal sensitivity was achieved with minimal ion delocalization, which was demonstrated at a 35 μm spatial resolution where we detected *N*-glycans characteristic of healthy glomeruli.[30]

Another recent advancement in *N*-glycan MALDI-MSI is incorporating METASPACE, an open-source FDR-controlled cloud engine for molecular annotation of MSI data, into the workflow.[27] Researchers can now automatically annotate and visualize their *N*-glycomics data using the specialized NGlycDB database (consisting of more than 3000 naturally occurring glycans), which is in METASPACE. In less than 10 minutes after submitting the data, annotated glycans and their ion images can be browsed, visualized,

searched, shared, and published. This image processing and molecular annotation step has previously taken many hours to days.

A limitation to spatial *N*-glycomics with MALDI-MSI is that sialic acids, which are labile decorations of glycans, are often lost in MALDI-MSI due to their in-source fragmentation from the parent glycan caused by the energy of MALDI laser.[31] This limitation cannot be understated, as the localization of sialic acids in the tissue is very informative. For example, these negatively charged moieties support the glomerular filtration barrier and maintain cellular structural integrity in the kidney, and glomerular hyposialylation is observed in severe pneumococcal infections, as well as in about 50% of patients with a sialic acid transporter defect.[32] Moreover, different anomerisms (i.e., different configurations of the anomeric carbon) of sialic acid linkages (i.e., α-2,6 or α-2,3) can be associated with different types of cancer.[33] As such, significant efforts have been made to stabilize sialic acids and differentiate sialic acid linkages in MALDI-MSI workflows through different on-tissue chemical derivatization (OTCD) strategies by converting the anomeric carboxylate group into an amide or ester that are less labile.[31, 34] Here, α-2,3 tends to form a six-member lactone ring that can be opened by a primary amine, whereas for α2,6-linked sialic acids, no lactonization reactions have been reported and a stable amide or ester is formed (depending on the reactant used).[34]

Besides *N*-glycans, MSI can also be used for imaging other types of glycans, such as large *O*-linked carbohydrate polymers on proteins (proteoglycans) that are essential components of the ECM in basement membranes and cell surface components on both endothelial cells and podocytes in the kidney.[35, 36] Herein, instead of PNGaseF specific for

N-glycans, other highly specific enzymes, such as Chondroitinase ABC, are sprayed to analyze glycosaminoglycan (GAG) chondroitin.[37] MSI of glycogen, an intracellular polysaccharide whose metabolism is very dynamic in the kidney, as it can be channeled to glycolysis and the Krebs cycle, is available through the *in situ* application of enzyme isoamylase. Detection of enzymatically released oligosaccharides enables insight into the frequency of glycogen branching across the tissue, informing on glycogen solubility and potency for pathological accumulation.[38] Naturally occurring, low molecular weight, unbound glycan structures can also be spatially profiled directly from the tissues without the need for enzyme application.[39, 40]

Imaging of glycans in kidney and kidney disease

There are great examples of utilizing lectin probes to reveal glycan epitopes associated with kidney cell functions, development, and pathology (**Figure 4)**. Thirty years ago, it was shown that jacalin lectin, specific for α-galactose, strongly stains the luminal border of distal tubules and single cells of the collecting tubules.[19] Even before this, researchers showed complicated glycan changes during kidney aging.[20] Notably, staining the superficial layer of the kidney cortex with *Lotus tetragonolobus* lectin for α-L-fucose was negative at the gestational stage but became positive with aging[20]. Furthermore, the reaction products for *Agaricus bisporus* lectin (ABA), specific for Gal and GalNAc, in the kidney cortex were localized mainly in the cytoplasm of proximal tubules and the blood capillaries of glomeruli during the late fetal period, and they were also consistently positive throughout development and aging.[20]

More recent glycan analysis using several lectins revealed glomerular epithelial cell hyposialylation, particularly the hyposialylation of podocalyxin, an important molecule for the glomerular filtration barrier.[41] Tomato lectin staining enabled visualization of high-mannose and polylactosamine *N*-glycans in the vascular structures of healthy kidneys,[18] and MoAb VIA 4.1 staining revealed that dystroglycan, a negatively charged glycoprotein that covers the surface of podocytes, was only decreased in lupus nephritis (LN) but not in minimal change nephropathy (MCN), focal segmental glomerulosclerosis (FSGS), and membranous glomerulopathy (MG).[42]

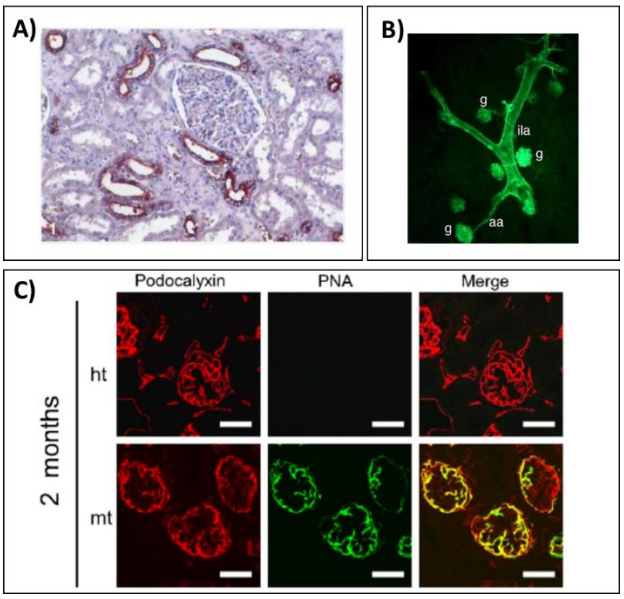

Figure 4. Lectin staining in kidney glycan research. A) Strong luminal staining of distal tubules by jacalin. Adapted from ref[19]. B) Kidney cortex, showing an intralobular arteriole (ila), afferent arterioles (aa), and several glomeruli (g). Adapted from ref[18]. C) Confocal laser scanning microscopic (scale bar 50µm) analysis of kidneys double-stained for PC

(podocalyxin, epithelial cell marker) and PNA lectin (specific for Galb1-3GalNAc) from 2-month-old heterozygous (ht) and mutant (mt) mice. Adapted from ref[41].

Emerging MALDI-MSI methods have helped reveal pathological and healthy kidney cell glycan structures and glycan-mediated processes (**Figure 5)**. For instance, spatial *N*-glycomics via MALDI-MSI revealed that in clear cell renal cell carcinoma, tumor regions have a low level of bisecting glycans and glycans with multiple fucoses compared to nontumor regions of the same tissue section. Moreover, in polycystic kidney disease, glycans in the cyst fluid regions lack fucose, while the same structures with fucose are localized to the healthy tissue regions[43]. In healthy kidneys, the fucosylated bisecting *N*-glycans and mannose structures localize to the cortex, whereas the predominant structural features of medullar glycans are basic biantennary and tri-antennary structures with a single fucose modification. A unique glycomic structure pattern is also associated with the interface between the cortex and medulla, represented by multifucosylated glycans lacking the bisecting GlcNAc.[43]

Analysis of sialic acid isomerism using OTCD revealed a significantly higher abundance of α2,6-linked sialic acid *N*-glycans in kidney cancer compared to healthy tissue, although there is no clear representation of where those sialic glycans are localized.[44] Alves *et al.* identified abnormal high mannose glycans distributed across all regions of the diseased kidney tissue in lupus nephritis patients.[45] This accumulation of mannosylated glycan structures resulted from a deficient *N*-glycosylation pathway that converges with an increased *O*-mannosylation pathway to generate mannose-enriched neoglycoantigens. Here, mannosylated glycans act as a glycan-epitope that can trigger the

recognition and activation of antigen-presenting cells through specific recognition by glycan-recognizing receptors, and oligomanose epitopes are typically found on the surface of several pathogens (i.e., virus, fungi, and parasites), representing an important trigger for immune activation[45].

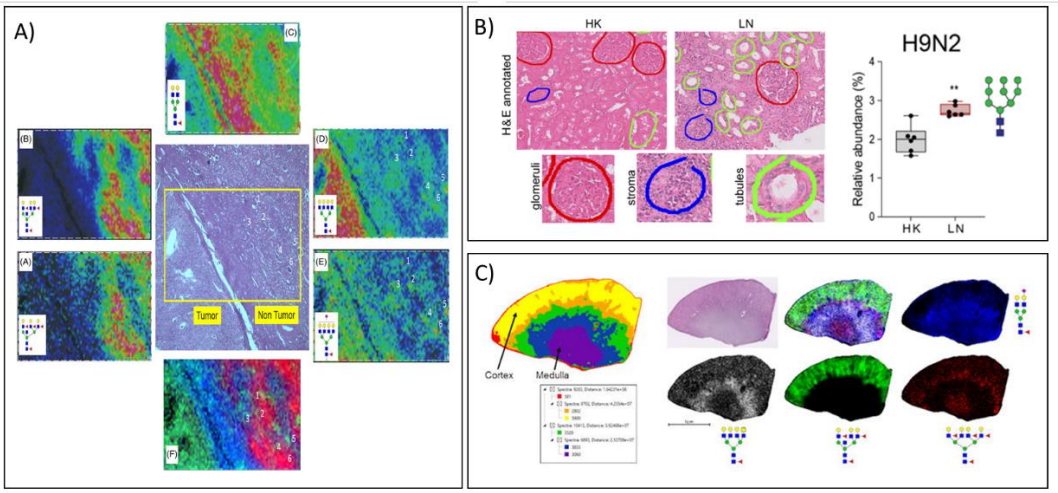

Figure 5. MALDI-MSI in kidney research. A) N-glycan profiles at the fibrillar interface between tumor and nontumor regions in clear cell renal cell carcinoma (ccRCC) Fuhrmann grade 2 tissue. Adapted from ref[43]. B) High mannose *N*-glycan (Hex:9 HexNAc2, H9N2) is highly abundant in Lupus nephritis (LN) kidney compared to healthy kidney (HK). Adapted from ref[45]. C) N-glycan profile of representative *N*-glycans in a cortex and medulla cross-section of a healthy kidney. Adapted from ref[43].

We have investigated glycan aberrations in sclerotic glomeruli of DKD patients and used a multi-omics combination of MALDI-MSI, single nuclei transcriptomics (snRNA-Seq), and spatial proteomics to better understand protein glycosylation processes in glomerulosclerosis and provide potential molecular markers of pathogenesis.[30] This was

possible due to the unique ability of the MALDI-MSI assay, which permitted going beyond simple epitope discovery and providing insight into the exact molecular composition of the detected *N*-glycan structures and their relative abundance throughout sections obtained from a needle biopsy at ~25 μm spatial scale. In total, 5 DKD patients and 3 healthy individuals were compared, resulting in 108 glomeruli with different degrees of sclerosis profiled. Twelve *N*-glycans were significantly enriched in glomeruli compared to other non-glomerular kidney parenchyma, all characterized by terminal galactose. *N*-glycans increased in sclerotic glomeruli did not contain fucosylation, and none of the *N*-glycans characteristic of sclerotic glomeruli have highly branched, tetra-antennary motifs. The most important finding is that the MS signal ratio between specific non-fucosylated (m/z 1976.6587, Hex:5 HexNAc:4 NeuAc:1) and the specific fucosylated glycan (m/z 2539.9037, Hex:7 HexNAc:6 dHex:1) can be used for distinguishing sclerotic and healthy glomerulus and even degree of glomerulosclerosis (**Figure 6)**.

Integration of MALDI-MS imaging molecular findings with other omics data from these patients (i.e., regional proteomic and snRNAseq) permitted gaining additional insights into glomerulosclerosis glycosylation and overcoming some of the MALDI-MS imaging limitations (e.g., spatial resolution and isomer differentiation). For example, snRNAseq ascribed branched *N*-glycans modification in sclerotic glomeruli to degenerative podocyte cell state, which would not be possible by MALDI-MSI alone due to insufficient spatial resolution. Further, regional proteomics data revealed that the sialic glycans identified in healthy glomeruli in the MALDI-MSI data are more likely the α-2,3

anomers, and that fucose is most likely linked to the core of *N*-glycans, resolving mass isomer ambiguities.[30]

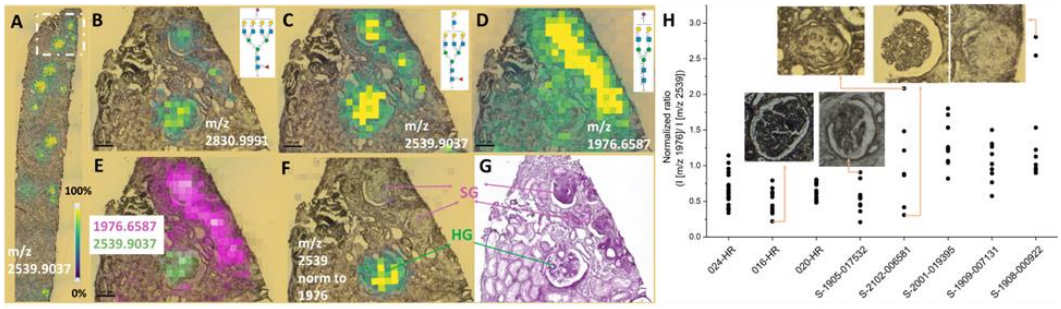

Figure 6. Example of high-resolution MALDI-MSI (pixel size 25µm) of *N*-glycans in DKD human biopsies. (A-G) MALDI images of selected N-glycans overlayed over microscopy images reveal their different abundance in healthy (HG) and sclerotic glomeruli (SG). (H) The normalized ratio of MS signal intensities at m/z 1976.6587 and m/z 2539.9037 in every glomerulus within the biopsies (multiple glomeruli per biopsy) as a measure of sclerotic severeness confirmed by examination of glomeruli appearance in microscopy images (inserts). Microscopy images of gloms with the specific normalized ratio are presented as inserts. Adapted from ref[30].

Conclusions and perspective

The emerging capacity to profile glycomes is of critical importance in molecular nephrology. Glycoconjugates serve as anchoring sites for cell adhesion, signaling receptors, immune cells, lectins, and pathogens, and glycan modifications are known markers of disease progression. Within the human glycome, *N*-glycans are the major class of carbohydrate modifications present in 90% of all glycoproteins. Recent studies have

linked specific glycosylation signatures with the progression of many forms of renal disease, including DKD, polycystic kidney disease, and renal cell carcinoma.

MSI is a prime technique for spatial glycomics because of its high sensitivity, high-duty cycle, and ability to provide structural information on glycans across the tissue at micrometer spatial scales. This makes it ideal for clinical applications, especially in biomarker discovery directly from the affected tissue. Although the adaption of MSI for glycomic analysis is barely a decade old and still evolving, at the moment of this review writing and related to kidney analysis, MSI revealed aberrant glycans in tumor regions of clear cell renal cell carcinoma, polycystic kidney disease, lupus nephritis patients, sclerotic glomeruli of DKD patients, and distinct glycans in different kidney functional and structural units in healthy human kidneys.

Nonetheless, two main challenges still need to be addressed to fully utilize the immense MSI potential in understanding and interpreting spatial glycobiology of human organs, including the kidneys. First, the spatial resolution of the MSI workflow is constrained to commonly ≥25 μm due to the laser ablation spot size, sensitivity issues, or unavoidable small delocalization of the glycans during the enzymatic digestion, which does not permit us to resolve the exact cellular origin of the glycan aberrations inside the functional tissue units (e.g., podocytes, mesangial cells, or endothelial cells inside glomerulus). Second, MSI alone cannot resolve glycans that have the exact same monosaccharide composition but different organization (mass isomers) because only the exact mass (i.e., mass to charge [m/z]) of the molecules is measured, so some ambiguity in the interpretation of glycans structure can occur. As such, improvements in the sample

preparation, sensitivity boost of glycans in MSI approaches, pre-mass analysis separations, and integration with orthogonal cell-specific modalities are expected in the near future to reveal exact glycan aberrations at the cell-specific level, directly linking to disease progression and glycan chemistry necessary for healthy kidney cell function.

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