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1 *Review*

2 **Kidney Lipidomics by Mass Spectrometry Imaging:** 3 **A Focus on the Glomerulus.**

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18 **Abstract:** Lipid disorders have been associated with glomerulopathies, a distinct type of renal
19 pathologies, such as nephrotic syndrome. Global analyses targeting kidney lipids in this
20 pathophysiologic context have been extensively performed, but most often regardless of the
21 architectural and functional complexity of the kidney. The new developments in mass spectrometry
22 imaging technologies have opened a promising field in localized lipidomic studies focused on this
23 organ. In this article, we revisit the main works having employed the Matrix Assisted Laser
24 Desorption (MALDI-TOF) technology, and the few reports on the use of Time Of Flight- Secondary
25 Ion Mass Spectrometry (TOF-SIMS). We also present a first analysis of mouse kidney cortex sections
26 by cluster TOF-SIMS. The latter represents a good option for high resolution lipid imaging when
27 frozen unfixed histological samples are available. The advantages and drawbacks of this developing
28 field are discussed.

29 **Keywords:** MALDI-TOF, TOF-SIMS, glomerulopathies, nephrotic syndrome, MSI
30

31 **1. Introduction: The Kidney Glomerulus as a Witness and Target of Lipid Disorders**

32 The renal glomerulus is a highly specialized anatomic structure containing the filtration barrier
33 that separates the blood and urine compartments. This barrier comprises a fenestrated endothelium,
34 a basement membrane, and a layer of podocytes, highly differentiated postmitotic epithelial cells.
35 Alterations in this barrier lead to proteinuria, which is one of the hallmarks of nephrotic syndrome
36 (NS), along with hypoalbuminemia, hyperlipidemia, lipiduria, and edema. NS characterizes a group
37 of diseases of either genetic or non-genetic—most likely immune—origin. The latter are known as
38 idiopathic nephrotic syndrome (INS), which comprehends two main histologically-defined forms,
39 namely minimal change nephrotic syndrome (MCNS) and focal and segmental glomerulosclerosis
40 (FSGS). Other glomerulopathies include the immune-mediated membranous nephropathy (MN), IgA
41 nephropathy, C3 glomerulopathies, lupus nephritis, anti-neutrophil circulating antibody-associated
42 vasculitis, and different forms of glomerulonephritis. In addition, a number of diseases, such as
43 diabetes, obesity and cancer, the exposure to infectious agents and toxic molecules, are associated
44 with glomerular dysfunction and secondary NS.

45 NS has been associated with a defect in lipoprotein metabolism and major changes in lipoprotein
46 profiling and content (reviewed in [1]). These alterations are attributed to impaired chylomicron and
47 very low-density lipoprotein (VLDL) clearance [2–4], which is associated, at least in part, with
48 decreased abundance of lipoprotein lipase, hepatic lipase, and glycosylphosphatidylinositol-
49 anchored binding protein 1 (GPI-BP1) in several tissues of animal models of NS. Other alterations
50 contributing to NS dyslipidemia are the high serum levels of angiopoietin-like protein 4 in NS
51 patients. The latter has been suggested to play a direct role in the onset of proteinuria [5]. Other
52 alterations include higher levels of cholesterol and LDL-cholesterol, due to increased synthesis and
53 decreased catabolism of cholesterol in the liver [6], and lower levels of HDL-cholesterol to cholesterol
54 ratio in NS patients [7]. Moreover, mutations in the *APOL1* gene, encoding apolipoprotein L1, a key
55 component of high-density lipoproteins (HDL), have been linked to FSGS susceptibility [8,9]. All
56 these alterations lead to higher risk of cardiovascular disease, and to local functional defects in the
57 kidney, such as nephron loss and lipotoxicity in proximal tubular cells. These observations parallel
58 the report of accumulated cholesterol in glomeruli from FSGS patients [10] and in the renal cortex of
59 diabetic nephropathy models [11]. Globally this suggests the presence of profound alterations in the
60 renal and the glomerular lipidome in these pathologic conditions.

61 Nevertheless, the glomerulus and the podocyte have been scarcely the target of global lipid
62 analyses [12,13]. In an elegant study, Jin and collaborators used a targeted lipidomic approach to
63 unveil the lipid interactors of soluble vascular endothelial growth factor (VEGF) receptor Flt1 at the
64 podocyte surface [13]. They identified the glycosphingolipid ganglioside M3 species as key
65 interactors, and this binding necessary to the functional integrity of podocyte and glomerular
66 filtration barrier. More recently, in a very different setup, lipidomic analysis of urine from pediatric
67 FSGS patients led to the conclusion that these individual display higher free fatty acids and
68 lysophosphatidylcholine (LPC) levels and lower phosphatidylcholine (PC) with respect to controls,
69 suggesting an increased phospholipase A2 activity associated with the FSGS glomerulus [12].

70 Several pathologies characterized by lipid disorders associated with glomerular alterations have
71 been described in the literature, such as Tay-Sachs, Gaucher, Niemann-Pick, Sandhoff, Fabry,
72 Myopathy 2, HIV-associated nephropathy, and diabetic nephropathy, among others. These disorders
73 are very often characterized by accumulation of different sphingolipid (SP) species (reviewed in [14]).
74 For example, in Fabry disease, the glycosphingolipids globotriaosylceramide (Gb3) and
75 digalactosylceramide (Ga2) significantly accumulate in several tissues, including the kidney, which
76 has been associated with podocyte dysfunction and proteinuria [15]. Sphingomyelin levels, along
77 with those of cholesterol, are increased in the kidney of Niemann-Pick disease, a genetic disorder due
78 to mutations in *NPC1/NPC2* (encoding intracellular cholesterol transporters) and *SMPD1* (encoding
79 an acid sphingomyelinase) genes, the latter associated with proteinuria [16]. Likewise, in genetic
80 forms of nephrotic syndrome due to mutations in the gene *NPHS1* (encoding nephrin, a protein
81 located at the podocyte slit diaphragm, a raft-like membranous structure), there is distal tubular and
82 parietal glomerular accumulation of disialoganglioside GD3 [17]. Moreover, post-transplant
83 recurrence of FSGS has been found linked to decreased expression of the acid sphingomyelinase-like
84 phosphodiesterase 3b (SMPDL3b) in podocytes, leading to increased sphingomyelin and decreased
85 ceramide [18]. Strikingly, in many of these pathologies the glycosphingolipid metabolic pathway is
86 altered.

87 2. Lipid Imaging of the Kidney: MALDI-MSI

88 Imaging analysis of kidney lipids by mass spectrometry imaging (MSI) is a relatively recent
89 approach that has made a still discreet yet significant contribution by accompanying histological
90 evaluation, global multiomic (including lipidomics) studies, and further proteomic analyses by MSI.
91 MSI lipidomics has extensively been performed on rodent material by matrix-assisted laser
92 desorption/ionization (MALDI), the mainstream method for MSI, which is characterized by the
93 addition of an organic chemical matrix on biological specimens. This technology has been used on
94 normal tissue [19,20–23] and in different pathophysiological contexts, such as acute renal injury [24],
95 diabetes/obesity [25], renal acidosis [26] polycystic kidney [27], drug-induced nephrotoxicity [28,29],

96 bisphenol toxicity [30], polymyxin toxicity [31], Fabry disease [32,33], and in glomerulopathies, such
97 as diabetic nephropathy [34] and IgA nephropathy [35].

98 Some of these studies have provided valuable information about the spatial localization of lipids
99 in kidney anatomical structures. One example is the thorough study that was performed to determine
100 the distribution of sulfated glycosphingolipids (sulfatides) by MALDI-MSI in combination with
101 source-decay LC-MS/MS in a mouse model of renal acidosis. The latter consisted of inactivation of
102 ceramide synthase, one of the key enzymes in the glycosphingolipid biosynthetic pathway. The
103 technical approach was able to discern the sulfatides characteristic of cortex, medulla, and papillae,
104 according to their sphingoid bases, C18-phytosphingosine, C18-sphingosine, and C20-sphingosine,
105 respectively. In particular, ceramide synthase deficiency was correlated with a depletion in C18-
106 phytosphingosine in the cortex region [26].

107 In other cases, although not strictly in the context of lipidomics, MSI has been employed to trace
108 an administered drug and its metabolites [28]. The reported study was performed on frozen and
109 paraffin sections of rabbit kidney, and the targeted small molecule, an inhibitor of c-Met tyrosine
110 kinase. This study was interesting in that samples were formaldehyde-fixed, and two technologies,
111 namely MALDI-MSI and DESI-MSI, were used. The authors examined cortex sections and identified
112 unambiguously crystal deposits containing the molecule and some of its metabolites in tubular areas.
113 This underlines the potential of MSI in this type of studies and also the feasibility of analyzing
114 previously-fixed paraffin material, even considering the ion suppression effects of paraffin and of
115 formaldehyde cross-linking.

116 2.1. MSI Analysis of Human Tissue: Lessons from Renal Cell Carcinoma

117 Much effort has been made in applying the spatial information provided by MSI techniques to
118 the study of human specimens in the context of kidney cancer, the rationale being the search for
119 differences in lipid composition between normal and tumor tissue, which could be used eventually
120 as diagnostic or prognostic markers. Recently, a MALDI variant consisting of gold nanoparticle-
121 enhanced target-based surface-assisted desorption/ionization (SALDI) has been performed to
122 analyze biopsies of renal cell carcinoma [36]. The study resulted in the identification of two markers
123 of tumor tissue, a sodium adduct of diglyceride DG 38:1 and protonated octadecylamide. Moreover,
124 MALDI-MSI was used in parallel to validate the results obtained by HILIC/ESI-MS when comparing
125 patient tumor material with adjacent normal tissue. By targeting polar lipids, such as gangliosides
126 and sulfoglycosphingolipids, and acidic phospholipids [37], MSI results confirmed the findings by
127 HILIC/ESI-MS, consisting of increased phosphatidylinositols, PI 40:5 and PI 40:4, and gangliosides,
128 GM3 34:1, and GM3 42:2, in carcinoma. In another study comparing normal and tumor tissue, MALDI
129 was coupled to an Orbitrap analyzer. The MALDI-Orbitrap method was initially focused on
130 sulfoglycosphingolipids and allowed the identification of more than 120 molecules. The use of
131 multivariate analyses led to the description of a discriminant signature and this correlated with the
132 result of the parallel MSI analysis. The latter showed two distinct markers, namely
133 phosphatidylethanolamine (PE) 36:4 and sulfodihexosyl-ceramide 42:1, decreased and increased in
134 carcinoma tissue, respectively [38]. Another study compared both types of tissues using two parallel
135 approaches, namely touch-spray ionization and DESI-MSI (desorption electrospray ionization-MSI).
136 Multivariate analysis enlightened a signature of discriminant lipid molecules encompassing ion
137 forms of phosphatidylserine (PS 36:1, PS 38:4) and phosphatidylinositol (PI 38:4) from MSI results,
138 while touch-spray led to discriminant phosphatidylcholine (PC)-derived ions [39].

139 In these examples, MSI often confirmed the results obtained by a LC-MS setup, but in some cases
140 unveiled new differences between both types of tissues, demonstrating the complementarity of both
141 strategies.

142 2.2. Glomerular Disease: The Spatial Limits

143 A few of those MSI studies targeting the kidney have attempted to address the lipid composition
144 of glomeruli, but the low resolution (i.e., most often in the 30–70 μm range), which roughly
145 corresponds to the mouse glomerular diameter, limits the observations to the renal cortex, as it is

146 unable to discriminate between glomerular and tubular structures. Nevertheless, some remarkable
147 findings have been reported to date.

148 One of these reports corresponds to a study on IgA nephropathy, the most prevalent glomerular
149 disease, characterized by deposition of immunoglobulin A on the glomerular mesangium. In a
150 spontaneous mouse model of the disease, the authors used a MALDI source coupled to two tandem
151 analyzers, a quadrupole ion trap, and a time-of-flight [35]. The analysis was able to distinguish the
152 renal cortex and the hilum, marked respectively by phosphatidylcholines (PC 38:6 and PC 40:6) and
153 triglycerides (TAG 52:3 and TAG 54:4). A signature characteristic of the diseased mice was composed
154 of PC O-38:6 and PC O-40:7 (ether forms of PC), an analog of platelet activating factor, and a
155 plasmalogen, respectively. In all cases, the presence of 22:6 (docosahexaenoate, DHA) moieties was
156 surprising, since this fatty acid is not known as particularly abundant in kidney tissue. In addition,
157 some ions were detected as characteristic of the IgA model, m/z 854.6, 856.6, 880.6, and 882.6, but
158 could not be identified. This was a pioneering study in several ways. On the one side, it is the first
159 reported MSI study focused on a glomerular disease. On the other side, the finding of a relevant
160 molecule containing a 22:6 moiety in the kidney, pointing at a particular function for this fatty acid
161 in this organ that is still remaining to be characterized. This is not an irrelevant issue, as in a chronic
162 kidney disease cohort study, low plasma levels of polyunsaturated fatty acids were found associated
163 with increased renal insufficiency [40]. Moreover, oral administration of n-3 fatty acids (DHA and
164 eicosapentaenoate, EPA) was shown to reduce proteinuria in FSGS patients [41], while the DHA
165 metabolite Resolvin D1 protected podocytes in an adriamycin-induced mouse model of glomerular
166 disease [42].

167 The latter (adriamycin/doxorubicin injection in rodents) represents one of the few available in
168 vivo models of nephrotic syndrome, as one single injection leads to the development of delayed
169 proteinuria. Hara and collaborators [43] combined doxorubicin injection with hypercholesterolemia,
170 in a mouse invalidated for LDL-receptor. Mice exhibited glomerular injuries resembling FSGS, with
171 podocyte alterations and foam cell infiltration in glomeruli. In this model, the authors found by
172 MALDI-MSI at an 80 μm resolution, two ions at m/z 518.3 and 543.3, identified as Na^+ adducts of
173 lysophosphatidylcholines LPC 16:0 and LPC 18:0, some of the main components of oxidized LDL,
174 that were abnormally increased in glomerular regions and colocalizing with oxidized phospholipids.
175 These molecules, when administered in vitro to cultured cells induced the expression of adhesion
176 molecules and cytokines, as well as adhesion and migration of macrophages. MSI in this study
177 contributed to unveiling an interesting lipid peroxidation mechanism associated with this mouse
178 model.

179 Lysophospholipids were also found increased in a mouse model of diabetic nephropathy, along
180 with PE, gangliosides, and sulfoglycosphingolipids (sulfatides) [34]. Glomerular and tubular lesions
181 accompany proteinuria and happen in a high proportion (about one third) of diabetic individuals.
182 The lipid changes reported in this study were, like in the FSGS model cited above, attributed, at least
183 in part, to increased oxidation. This was proven by the use of pyridoxamine, an inhibitor of oxidative
184 processes, as a control condition. The changes were presented as happening in glomeruli and/or
185 tubuli, in spite of a relatively high resolution (between 10 and 40 μm). Interestingly, ganglioside GM3
186 forms, m/z 1151.7 (NeuAc-GM3) and m/z 1167.7 (NeuGc-GM3), showed a pointed pattern in the
187 kidney, specifically colocalized with glomeruli (identified by PAS staining). NeuGc-GM3, the
188 oxidized form, was found increased in diabetic kidneys. The same pattern was found for
189 sulfoglycolipid SB1a t18:0/22:0, m/z 1407.8, as compared with its non-oxidized form, as well as
190 glucose-modified PE (Amadori-PE, a plasma marker of diabetes). In all cases the increase was
191 corrected with the pyridoxamine treatment. Modified PE; however, was not specific of glomerular
192 regions. These outstanding results indicate that GM3 and sulfated sphingolipids are particularly
193 abundant in glomeruli, most likely in podocytes, and point at this particular lipid class as a target of
194 glomerular alterations in pathologic conditions.

195 Modifications in the lipid content of glomeruli in diabetes has been the result of another study,
196 devoted to a MALDI-MSI metabolomic profiling of diabetic mice exposed to high-fat diet. In this case
197 the initial search for nucleotide distribution in tissues ended up in a lipid finding. Thus, along with

198 the finding of an altered ATP/AMP ratio, untargeted analysis revealed an accumulation of
199 sphingomyelin SM d34:1 in the glomeruli of those mice as compared to controls [25]. It was argued
200 that the sphingomyelin accumulation could be responsible for the increased ATP production, due to
201 activation of the glycolytic pathway. In this study, the results in glomeruli were obtained with a
202 relatively high-resolution setting (30 μm). Glomeruli were identified using a phosphatidic acid
203 species (PA 36:1) as a glomerular signature. This represents an interesting strategy to be used in the
204 selection of regions of interest in those MSI studies addressing glomerular lipid composition.

205 Another condition that leads to glomerular dysfunction is renal toxicity due to bisphenol
206 exposure. Experimentally, bisphenol targets glomeruli inducing necrosis, in addition to other lesions,
207 such as cloudy swelling of medulla and interstitial collapsing of renal pelvis, in mice exposed to
208 different concentrations of the compound [30]. The modifications in the cortex were associated with
209 differential distribution of seven lipids, including accumulation of SM d22:0/20:4, TAG 16:0/14:0/16:0,
210 PS 18:0/22:6, and PG 16:0/16:0 and a decrease in DAG (18:0/22:6), PE (20:1/20:4), and PI (16:1/18:1)
211 when compared with control mice. The results suggest that the renal cortex is the most sensitive area
212 to the toxic, and a 2D and 3D model construction coupled with a multivariate analysis pointed to a
213 signature specific of bisphenol exposure, based on increased SM d22:0/20:4 and Cer d18:2/24:1, which
214 could eventually be considered as a marker of toxicity.

215 Finally, a matrix-free variant of MALDI, such as NALDI-MSI has been used to target renal lipids
216 in normal mouse kidney [44], showing increased presence of a K^+ adduct of PC 32:0 in the renal cortex
217 as compared to the medulla [45]. NALDI technology, in this case consisting of a Fourier transform
218 ion cyclotron resonance mass spectrometer, was applied in parallel to study the brain and kidney,
219 and compared with MALDI. The authors claimed NALDI to improve mass accuracy and detection
220 efficiency. It could represent an alternative to conventional MALDI-MSI in kidney-based studies.

221 3. The TOF-SIMS Alternative

222 Imaging by cluster time-of-flight secondary ion mass spectrometry (TOF-SIMS) represents a
223 powerful tool for localized lipidomics. SIMS was first developed in the 1960s and 1970s for surface
224 analysis [46,47]. This technology consists of the bombardment of the sample by a beam of polyatomic
225 ions, which induces desorption/ionization of secondary ions from the sample surface. Polyatomic ion
226 focused beams have been used successfully in the analysis of organic surfaces [48–53]. The current
227 development of the technology allows a high lateral resolution of some hundreds of nanometers,
228 which makes the technology particularly well fitted for the analysis of tissue sections [48,52–62]. In
229 addition, no matrix is required for ion generation, which avoids all the drawbacks associated with
230 matrix deposition on tissue.

231 The analysis of kidney tissue by TOF-SIMS was first performed in rats by gold particle
232 bombardment on silver-coated sections [63–65]. In their first report, the authors found a two-fold
233 improvement in sensitivity by silver-coating and attained a 200 nm lateral resolution. They reported
234 a clear patchy distribution of silver-cationized cholesterol ions (m/z 493.4 and m/z 495.4). These
235 cholesterol-rich areas were identified as nuclear regions of distal tubular epithelial cells [64]. In
236 another report they attributed high cholesterol levels to glomerular areas [63]. In their third work,
237 Na^+ ions were found increased in glomerular areas, while phosphocholine (m/z 184) was uniformly
238 localized, along with K^+ ions.

239 The only report so far of TOF-SIMS analysis of human kidney biopsies was performed by our
240 group in the context of Fabry disease [66], an X-linked disorder characterized by accumulation of Gb3
241 and Ga2 in the renal cortex due to a deficiency in α -galactosidase A. Our approach was able to detect
242 both sphingolipid molecules characteristic of this pathology in human material [67]. More recently, a
243 MALDI-MSI approach has detected the same defect in the kidney of α -galactosidase A knockout mice
244 [33]. Apart from these pioneering studies, no systematic analysis of glomerular lipids has been
245 addressed by TOF-SIMS imaging.

246 4. Cluster-TOF-SIMS analysis of mouse kidney cortex

247 Here we report the use of cluster-TOF-SIMS to analyze the kidney cortex of normal mice. Three-
248 month old C57B6 mice were sacrificed, kidneys were harvested, embedded in optimal cutting
249 temperature (OCT), and immersed in frozen in liquid nitrogen at $-195\text{ }^{\circ}\text{C}$. Samples were stored at -80
250 $^{\circ}\text{C}$. Before analysis, $14\text{ }\mu\text{m}$ -thick tissue sections were obtained at $-20\text{ }^{\circ}\text{C}$ using a cryostat (CM3050-S
251 Cryostat LEICA Microsystems, SAS, Nanterre, France), then deposited onto a conductive indium thin
252 oxide (ITO) slide and dried for 15 min under a pressure of a few hectopascals.

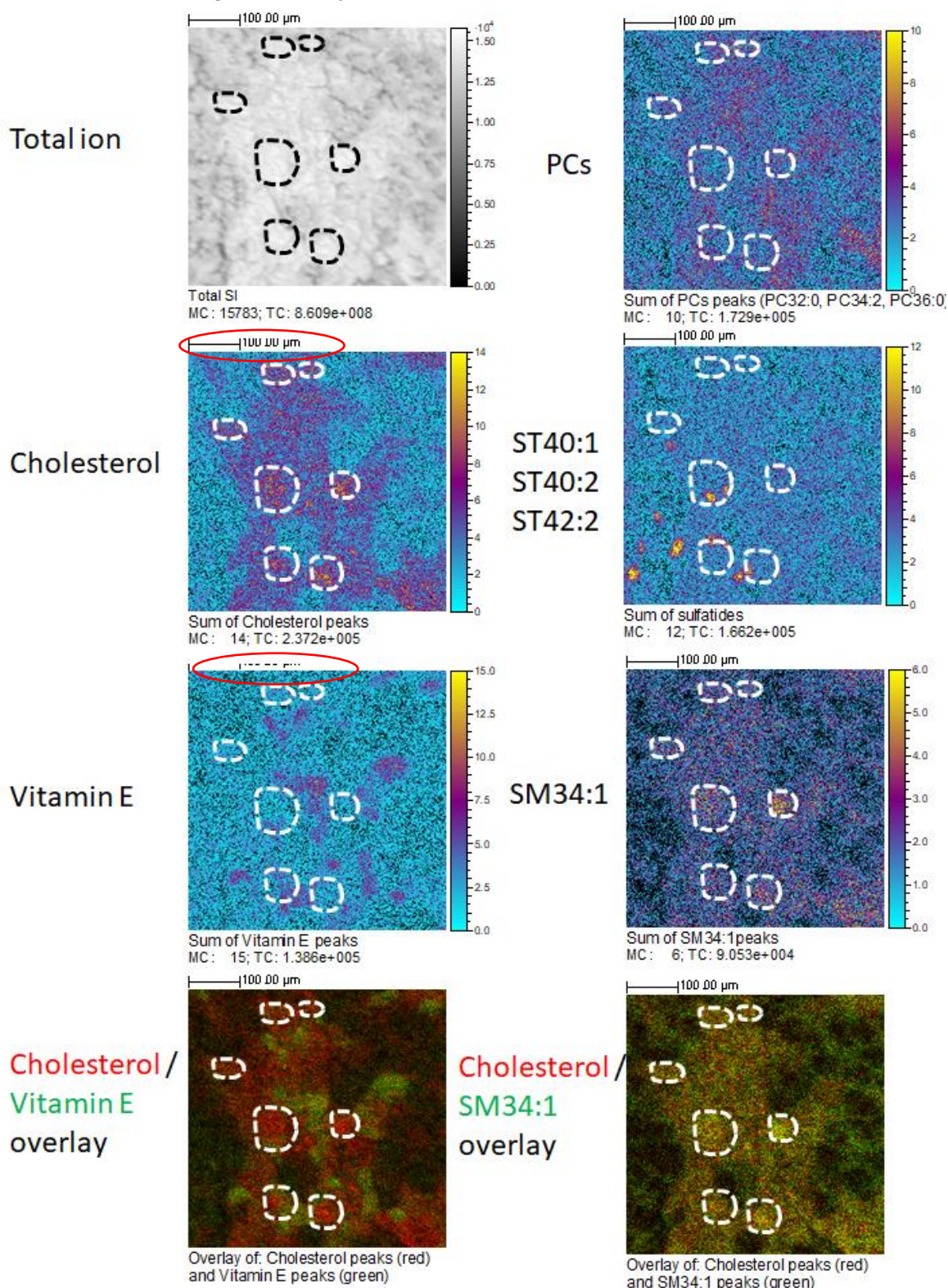
253 We used a TOF-SIMS IV mass spectrometer (ION-TOF GmbH, Münster, Germany) with bismuth
254 ion source and a reflectron time of flight analyzer. Bi_3^+ cluster primary ions hit the surface of the tissue
255 section with a kinetic energy of 25 keV and an incidence angle of 45° . The focusing mode which is
256 used are the so-called “high current bunched” mode. Secondary ions are extracted with an energy of
257 2 keV and are post-accelerated to 10 keV just before hitting the detector. A low-energy electron flood
258 gun is used to neutralize the surface during the experiments. For each sample, two areas of 500×500
259 μm with 256×256 pixels were analyzed with a primary ion dose of 2×10^{12} ions/ cm^2 . The stability of
260 the ion emission of different components is controlled along the analysis, most of ions kept the same
261 intensity during the analysis, so that the analysis can be considered as being done below the so-called
262 static limit. An internal mass calibration has been made with H^+ , H_2^+ , H_3^+ , CH_3^+ , and $\text{C}_{29}\text{H}_{50}\text{O}_2^+$
263 ([Vitamin E] $^+$) ions in the positive ion mode and with H^- , C^- , CH^- , C_2^- , C_2H^- , C_3^- , C_3H^- , C_4^- , C_4H^- , and
264 $\text{C}_{29}\text{H}_{49}\text{O}_2^-$ ([Vitamin E-H] $^-$) ions in the negative ion mode. On each ion image, “MC” represents the
265 maximal number of counts in a pixel and “TC” the total number of counts. The color scales
266 correspond to the interval $[0, \text{MC}]$.

267 The global spectra of glomerular and non-glomerular areas were compared. A total of 136
268 distinct ions (67 in the positive ion mode and 69 in the negative ion mode) were detected and
269 attributed with one or more potential identities by comparison with the m/z values of bona fide
270 standards and/or compared with standards in the ION-TOF database (<https://www.iontof.com>).
271 Some of these standards have been analyzed in our laboratory and the spectra published [51,57,60,68].
272 Examples of standard spectra are shown in Figure S1. The m/z and attributed identities are presented
273 in Supplementary Table S1 and Table S2. Among these ions, 128 were identified as lipids, lipid
274 adducts or lipid fragments, belonging to six different lipid categories (according to Lipid MAPS
275 classification). All of them were detected both in glomerular and non-glomerular areas.

276 Figure 1 shows the relative intensity maps of five ions or ion combinations corresponding to the
277 sum of cholesterol peaks, of SM 34:1 peaks, α -tocopherol (vitamin E) peaks, sulfatides ST 40:1, ST
278 40:2, ST 42:2, and PC 32:0, PC 34:2, PC 36:0. Relative intensity is represented by a color scale, in which
279 yellow corresponds to higher and blue to lower abundances. Glomerular areas were surrounded by
280 a white dashed line. We observed a consistent increased intensity of both cholesterol ions and SM
281 34:1 in glomerular areas. In the lower panels, one artificial color (red or green) was attributed to each
282 compound, which allowed visualizing the co-localization of either cholesterol and α -tocopherol or
283 cholesterol and SM 34:1. As it can be observed, cholesterol co-localized with SM 34:1 and counter-
284 localized with α -tocopherol (yellowish signal) in all cortical tissue, but intensity was higher in
285 glomerular areas. Sulfatides presented a distinct patchy distribution in non-glomerular regions, while
286 PC ions were distributed more or less homogeneously throughout the renal cortex, but showing a
287 slightly higher abundance (red color) in non-glomerular areas. Figure 2 shows the distribution of
288 cholesterol (in positive and negative mode) in glomerular and non-glomerular regions.

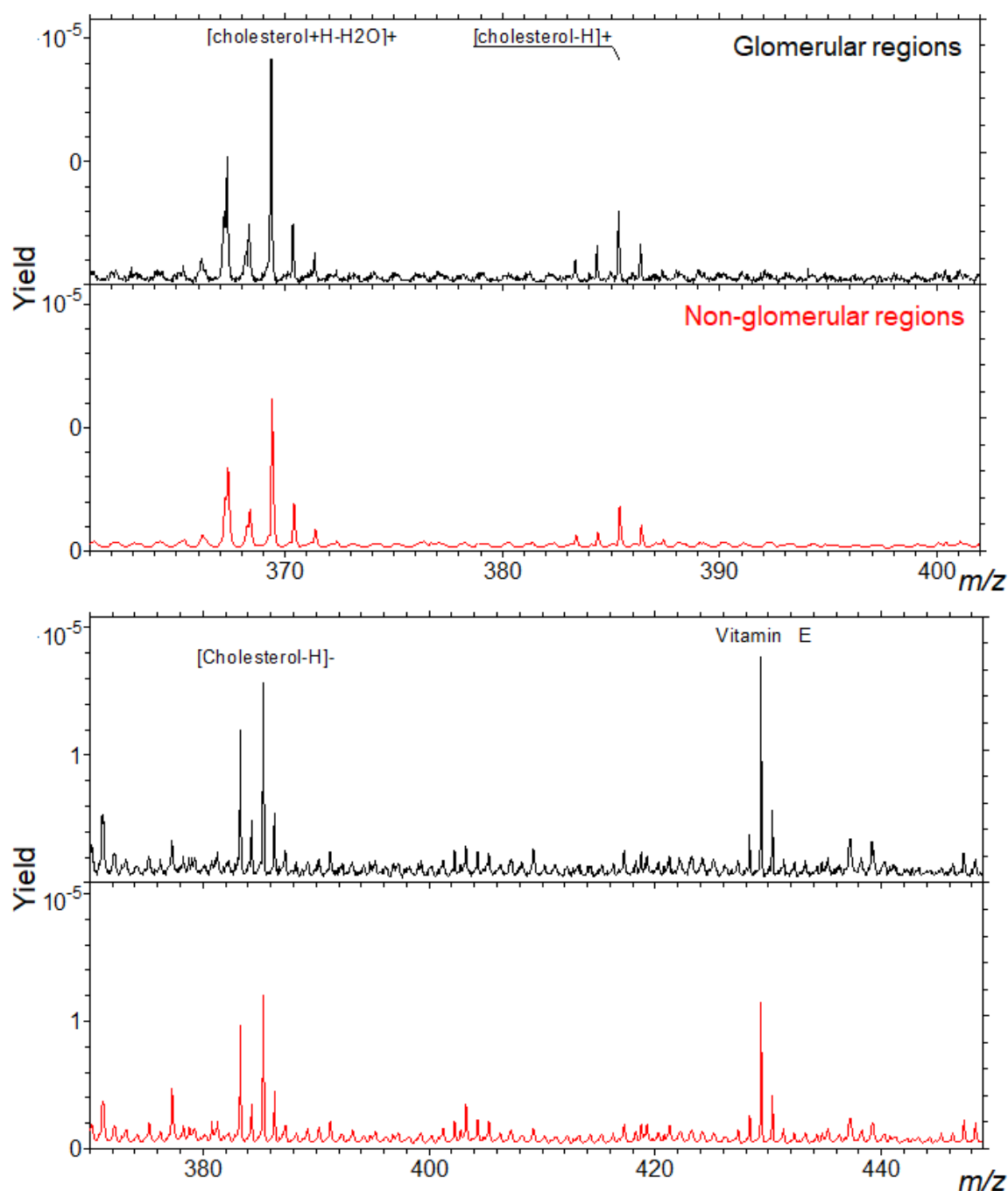
289 In agreement with our result, a MALDI-MSI-based analysis of rat kidney combined with
290 histological data has reported cholesterol and squalene as specific glomerular metabolites [19].
291 Cholesterol is; therefore, enriched in glomeruli, but not exclusively. Additionally, we found an
292 enrichment in SM d34:1 in glomerular regions, which could correspond to the same molecule that
293 has been found increased in the glomeruli of a mouse model of diabetic nephropathy in comparison
294 with control animals [25]. Ideally, an exclusive ion could be used for ROI selection in only-glomerular
295 studies. The studies by other groups have proposed interesting glomerular markers, such as PA
296 (36:1). Unfortunately, our TOF-SIMS setup was unable to detect this ion. Glomeruli are, nevertheless,
297 an anatomical structure relatively easy to identify in adjacent sections by conventional staining, and
298 even on ion images. Lipid ions found in glomerular areas can reflect the lipid composition of

299 podocytes, parietal epithelial cells, or glomerular endothelial cells. Our setup can be used to establish
 300 modifications in the lipid composition of glomeruli in mouse models of glomerular diseases or in
 301 biopsies from patients. Potential changes found in these areas would be complementary to those
 302 found in global lipidomic analyses on isolated glomeruli, or on primary podocytes. The setup
 303 presented above provides the combination of a good spatial resolution, the unbiased identification
 304 of a large number of molecules, and the potential for glomerular ROI selection and subsequent
 305 relative quantitation. This setup will be used to explore variations in the localization and intensity of
 306 these ions in models of glomerular dysfunction.



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Figure 1. Representative section of a mouse kidney cortex, showing the spatial distribution of several ions (sum of cholesterol peaks, sum of α -tocopherol peaks, sum of phosphatidylcholine (PC) peaks, sum of sulfatides ST 40:1, ST 40:2, ST 40:3, sum of sphingomyelin (SM) 34:1 peaks, and the total ion current) at a spatial resolution of 2 μm per pixel. The scale on the right (gray levels or colors) indicates the relative abundance. Yellow or white denote higher abundances, while blue or black denote lower abundances. The two lower panels have been constructed by attributing one color (red or green) to each compound. Glomerular regions of interest are selected and depicted by the dashed white line. Bar length: 100 μm . The name of the compound, the maximal number of counts in a pixel (MC), and the total number of counts (TC) are written below each image.



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Figure 2. Representative spectra of a mouse kidney cortex, showing the relative intensities of peaks corresponding to cholesterol ions (in positive-top- and negative-bottom-mode) in glomerular and non-glomerular regions. The spectra are normalized to the total primary ion dose.

321 5. Concluding Remarks: Possible Evolution of Kidney MSI. Application to Glomerulopathies

322 The advantages of MSI are, mainly, the possibility of a multiplexed analysis of several ions in a
 323 single acquisition, and the information about their spatial localization. The drawbacks, the
 324 insufficient resolution as compared to immunofluorescence and electron microscopy, and the limited
 325 availability of technical platforms, especially TOF-SIMS. Nonetheless kidney MSI has been proven to
 326 support and complement other untargeted lipidomic studies.

327 INS, and in particular MCNS and FSGS, unlike other glomerulopathies, require renal biopsy to
 328 establish the right diagnosis. Therefore, these forms are defined histologically, meaning that little
 329 information is provided by regular optical microscopy evaluation of tissue sections. MSI could be
 330 helpful both in diagnosis, and as an unbiased way of finding molecular mechanisms that could clarify
 331 their uncertain pathogenesis. To date, two main technical approaches for MSI of lipids have been
 332 used in the context of renal disease. MALDI-TOF and TOF-SIMS cannot be considered as competing
 333 approaches, since they can provide complementary information at different levels (Table 1). Firstly,
 334 the type of compounds covered by TOF-SIMS are under m/z 1500, including small molecules, lipids,
 335 metabolites, and elements, while the MALDI-TOF range is over m/z 200, which includes many lipids
 336 along with peptides. TOF-SIMS presents some advantages, like a higher spatial resolution, higher
 337 sensitivity for low molecular weight compounds, no need for homogeneous matrix coating, and the
 338 potential for 3D mapping. However, it also presents some drawbacks, mainly associated with large
 339 fragmentation, often making identification of original molecules problematic. MALDI-TOF is;
 340 therefore, more suitable for unambiguous compound identification. In both cases the analysis is
 341 relatively long and complex, and always semiquantitative.

342 **Table 1.** Comparison of the features provided by TOF-SIMS and MALDI-TOF in the context of MSI.

	TOF-SIMS	MALDI-TOF
Analysis	Elemental and molecular analysis	High molecular weight covering a large range of molecules
Compounds	Lipids, glycosphingolipids, cyclopeptides, drugs, metabolites, minerals	Proteins, peptides, lipids, drugs, metabolites
Mass range	$m/z \leq 1500$	$m/z > 200$
Sample	Dehydrated, no fixation, no matrix	Dehydrated, homogeneous matrix coating
Imaging	Elemental and chemical imaging and mapping in 2D and 3D	Possibility to characterize and visualize in 2D
Spatial resolution	Down to 100–400 nm	5–50 μm
Sensitivity	High sensitivity for trace elements or compounds, in order of ppm to ppb for most species	Low sensitivity for low molecular weight molecules
Overall	Long and complex semi-quantitative analysis	Long and complex semi-quantitative analysis

343 New developments in MALDI-MSI aim at increasing spatial resolution. An example is a
 344 reported atmospheric-pressure (AP-MALDI) setup applied to brain and kidney imaging, leading to
 345 a lateral resolution of 1.4 μm and coupled to an Orbitrap analyzer [69]. This setup has efficiently
 346 shown the tissue localization of PC 38:6, PC 40:6, PC 38:1, PC 32:0, PS 40:0, and SM d36:0. On the
 347 other side, recent developments in TOF-SIMS aim to give access to a much higher mass resolution,
 348 with the recent launch of the Orbi-SIMS instrument [70] to in situ structural analysis of secondary
 349 ions through a tandem TOF analyzer (TOF/TOF) [71].

350 Interestingly, most of the studies published so far on kidney MSI point at alterations of the
 351 glycosphingolipid profile in different pathologies involving glomerular dysfunction (Table 2). This
 352 lipid class seems to play a significant role in the correct function of the organ, and of the glomerular
 353 barrier in particular. In fact, gangliosides are especially abundant at the surface of podocytes [13].

354 Integrity of the slit diaphragm, a lipid raft-like membranous structure establishing the interaction
 355 between foot processes of adjacent podocytes, is a key aspect in filtration barrier function.
 356 Sphingolipids are main components of this structure, and their metabolism seems to play a major
 357 role. In our setting, we have found an increased presence of a sphingomyelin species (SM d34:1) in
 358 mouse glomeruli. Nevertheless, other relevant lipid classes have been identified throughout MSI
 359 lipidomic analyses, such as glycerophospholipids and glycerol-lysophospholipids, and, most
 360 importantly, their differential esterifying acyl chains have been unambiguously identified.
 361 Glomerular lipidomics is still underexplored, and MSI constitutes a promising strategy to address
 362 this point.

363 **Table 2.** Synthesis of reported findings by lipid MSI in renal cortex and glomerular areas.

reference	species	Technology	Pathology/condition	Main finding (ion/molecule)	Lipid MAPS category	Anatomical region
[30]	mouse	MALDI	Bisphenol toxicity	SM d22/20:4 Cer d18:2/24:1	SP	Cortex
[35]	mouse	MALDI	IgA nephropathy	PC O-16:0/22:6 PC O-18:1/22:6	GP	Cortex
[34]	mouse	MALDI	Diabetic nephropathy	NeuGc-GM3	SP	Glomeruli
[25]	mouse	MALDI	Diabetic nephropathy	SM d18:0/16:0	SP	Glomeruli
[28]	mouse	MALDI	FSGS (doxorubicin injection model)	LPC 16:0 LPC 18:0	GP	Glomeruli
[33]	mouse	MALDI	Fabry disease model	Gb3; Ga2	SP	Glomeruli
[66]	Human biopsies	TOF-SIMS	Fabry disease	Gb3; Ga2	SP	Glomeruli
[44] [45]	mouse	MALDI	normal	PC 32:0	GP	Cortex
[25]	mouse	MALDI	normal	PA 36:1	GP	Glomeruli
[63,64]	mouse	TOF-SIMS	normal	Cholesterol	SL	Glomeruli
[65] this report	mouse	TOF-SIMS	normal	Cholesterol SM d34:1	SL-SP	Glomeruli

364 **Supplementary Materials:** Supplementary materials can be found at www.mdpi.com/xxx/s1. Table S1: Negative
 365 ions detected by TOF-SIMS analysis of mouse renal cortex. Table S2: Positive ions detected by TOF-SIMS analysis
 366 of mouse renal cortex. Figure S1: Upper panel, partial positive spectrum of phosphatidylcholine PC32: 0
 367 standard from Sigma. Lower panel, partial negative spectrum of sulfatide 42:2(18:1/24:1) standard from Sigma.
 368 The analyses were done at ICSN-CNRS laboratory. Experimental spectra were compared with standard spectra
 369 for identity attributions.

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377 Abbreviations

Cer	Ceramide
DHA	Docosahexaenoate
DESI	Desorption electrospray ionization
GL	Glycerolipid class
GP	Glycerophospholipid class
GPI-BP1	Glycosylphosphatidylinositol-anchored binding protein 1
HILIC	Hydrophilic interaction chromatography

INS	Idiopathic nephrotic syndrome
LC-MS	Liquid chromatography-mass spectrometry
LPC	Lysophosphatidylcholine
MALDI	Matrix-assisted laser desorption ionization
MCNS	Minimal change nephrotic syndrome
MN	Membranous nephropathy
MSI	Mass spectrometry imaging
NALDI	Nanostructure-assisted laser desorption ionization
NS	Nephrotic syndrome
PC	Phosphatidylcholine
PR	Prenol lipid class
SALDI	Surface-assisted laser desorption ionization
SIMS	Secondary ion mass spectrometry
SL	Sterol lipid class
SM	Sphingomyelin
ST	Sulfatide
SP	Sphingolipid class
TOF	Time of flight
VLDL	Very low-density lipoprotein

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