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1 *Review*

## 2 **Gangliosides in podocyte biology and disease**

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11 **Abstract:** Gangliosides constitute a subgroup of glycosphingolipids characterized by the presence  
12 of sialic acid residues in their structure. As constituents of cellular membranes, in particular of raft  
13 microdomains, they exert multiple functions, some of them capital in cell homeostasis. Their  
14 presence in cells is tightly regulated by a balanced expression and function of the enzymes  
15 responsible for their biosynthesis -ganglioside synthases- and their degradation -glycosidases-.  
16 Dysregulation of their abundance results in rare and common diseases. In this review we make a  
17 point on the relevance of gangliosides and some of their metabolic precursors, such as ceramides,  
18 in the function of podocytes, the main cellular component of the glomerular filtration barrier, as  
19 well as their implications in podocytopathies. The results presented in this review suggest the  
20 pertinence of clinical lipidomic studies targeting these metabolites.

21 **Keywords:** glycosphingolipids; ceramide; podocytopathies; glomerulopathies; glomerulus; kidney;  
22 rafts; nephrotic syndrome  
23

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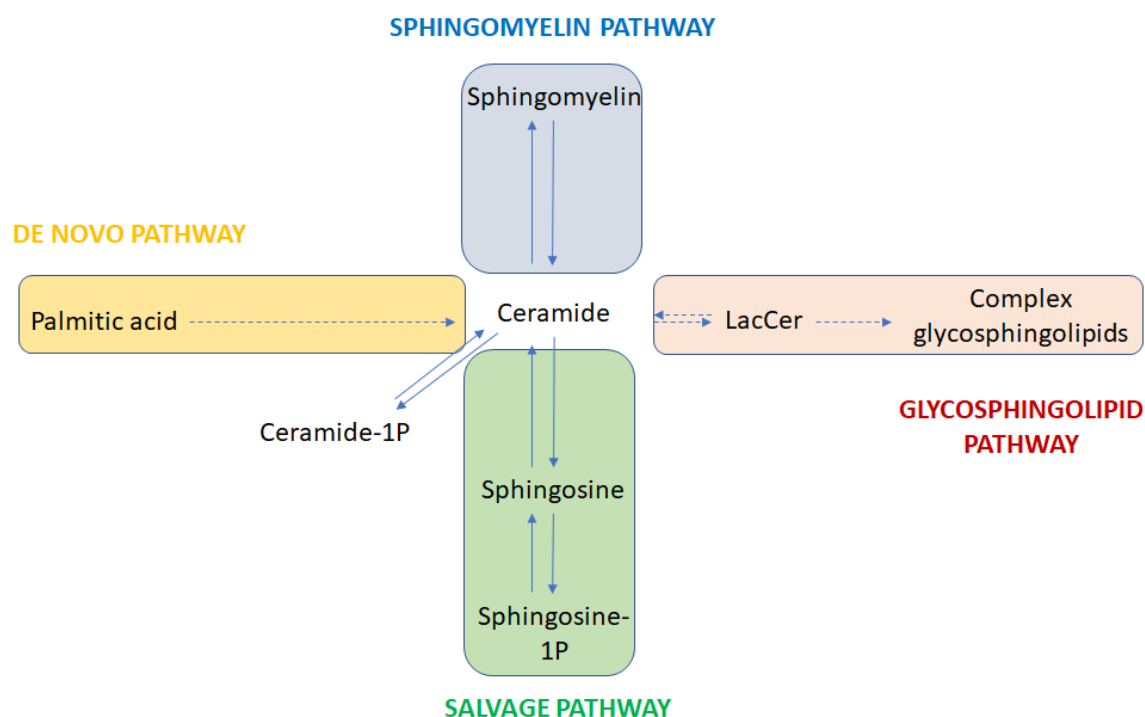
### 24 **1. An Overview of Sphingolipid and Glycosphingolipid Metabolism**

25 Sphingolipids represent one of the eight categories that currently the Lipid MAPS consortium  
26 classifies as lipids. They are characterized by the presence of a sphingoid base backbone, consisting  
27 of an aliphatic long chain amino alcohol, most frequently represented by sphingosine. Sphingolipids  
28 constitute a highly heterogeneous category of molecules, with a central group of compounds,  
29 ceramides, containing a fatty acid moiety associated with the sphingoid base via an amide bond [1].  
30 The high diversity of sphingolipids is the result of four different anabolic and catabolic pathways  
31 that converge and diverge to and from the ceramide backbone (Figure 1). These are known as the  
32 “de novo” synthesis, the hydrolytic, the sphingomyelin and the catabolic or salvage pathways.

33 Ceramides are highly versatile compounds that can undergo glycosylation, among other  
34 modifications. Glycosylation of ceramides through the so-called hydrolytic pathway generates the  
35 glycosphingolipid category, encompassing several hundred compounds. Ceramide glycosylation  
36 leads to glucosylceramide (GlcCer), then through galactosylation to lactosylceramide (LacCer).  
37 LacCer is the ultimate precursor of complex glycosphingolipids, namely globosides, cerebrosides  
38 and gangliosides. Within glycosphingolipids, gangliosides are defined by the presence -with some  
39 exceptions- of at least one sialic acid residue -a derivative of neuraminic acid- associated with an  
40 oligosaccharide chain. A 5-N-acetyl derivative of neuraminic acid is the most abundant form of sialic  
41 acid in humans, with 10% corresponding to the 5-N-acetyl-9-O-acetyl derivative [2]. Three main  
42 pathways produce all gangliosides (Figure 2). Monosialylated ganglioside M3 (GM3) is the simplest  
43 molecule in the pathway, and precursor of all gangliosides of the a-, b-, and c- series. It contains one  
44 single sialic acid residue and is produced by sialylation of LacCer by GM3 synthase (ST3Gal5) [3].

45 An excellent description of ganglioside structure, structural variability, and their implications for  
 46 interaction with other membrane molecules is provided in the publication by Mauri and colls. [1].

47

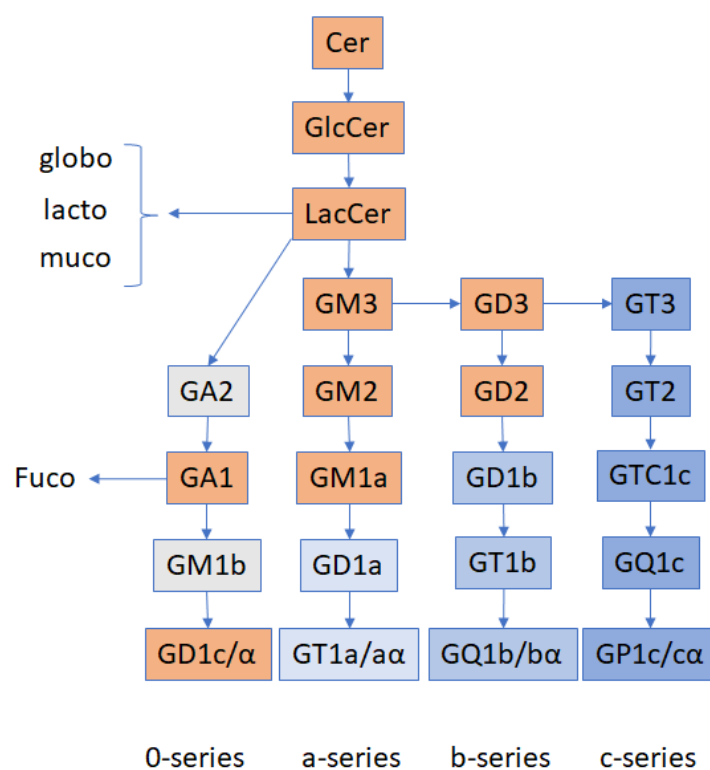


48

49 **Figure 1.** Global view of sphingolipid metabolism. Simplified representation of the four main  
 50 pathways encompassing the synthesis of sphingolipid species, with ceramides as central  
 51 compounds. Plain arrows indicate single reactions. Dashed arrows denote multiple reactions. De  
 52 novo and sphingomyelin pathways result in ceramide synthesis from palmitic acid and  
 53 sphingomyelin as ultimate precursors respectively. The so-called salvage or catabolic pathway  
 54 results in the production of bioactive sphingosine-1 phosphate. Ceramide itself can be  
 55 phosphorylated into the bioactive ceramide-1 phosphate. Finally, the hydrolytic or  
 56 glycosphingolipid pathway leads to ceramide glycation. Not shown on the figure,  
 57 galactosyl-ceramide is the precursor of sulfatides. The rest of complex glycosphingolipids  
 58 (lactosides, globosides, cerebrosides and gangliosides) are derived from lactosyl ceramide (LacCer).  
 59 All pathways are reversible except de novo synthesis.

60 Gangliosides are particularly abundant in cellular membranes, due to the hydrophobicity of  
 61 their ceramide backbone. Their presence is highly related to membrane function and associated with  
 62 cell homeostasis, as they are among the most abundant and characteristic components of the  
 63 specialized microdomains defined as lipid rafts. Their hydrophilic heads are much bulkier than  
 64 those of glycerophospholipids, this feature determining their lateral separation properties, resulting  
 65 in aggregation and generation of a positive curvature [1].

66 Lipid rafts or liquid-ordered microdomains are very rich in lipids containing saturated or little  
 67 saturated acyl chains in their hydrophobic moieties [4]. Ceramides and their glycosylated derivatives,  
 68 gangliosides, correspond to this description, and consequently they are some of the main lipid  
 69 components of rafts. Ganglioside function in the cell is, therefore, associated with their chemical  
 70 structure, lateral mobility, and their ability to partition in raft-like environments.



71

72 **Figure 2.** Schematic representation of ganglioside biosynthesis pathways. LacCer are the precursors  
 73 of the globo, lacto, muco and ganglio series of glycosphingolipids. GM3 are the precursors of a, b and  
 74 c series of gangliosides. LacCer are precursors of asialo (0-series) gangliosides (GA). GA1 give way to  
 75 fucosylated glycosphingolipids. “/” denotes two different structures of gangliosides produced from  
 76 the same precursor. “α” denotes specific ganglioside structures in which one sialic acid residue is  
 77 branched to N-acetylgalactosamine (GalNAc). All other sialic acid residues are branched to galactose  
 78 residues (modified from [5]). Orange colored rectangles denote molecular species whose abundance  
 79 has been reported to date as changed in the context of podocytopathies (summarized in Table 1).  
 80 Many of these reactions are reversible. GM: monosialo gangliosides. GD: disialo gangliosides. GT:  
 81 trisialo gangliosides. GQ: quadrisialo gangliosides. GP: pentasialo gangliosides.

82 As key membrane components, gangliosides are involved in the regulation of cell signaling,  
 83 intercellular crosstalk, pathogen invasion, apoptosis/survival, proliferation/differentiation and  
 84 immune cell function. Such a diverse array of functions implies a tight regulation of their anabolism  
 85 and catabolism, whose dysfunction results in a number of pathologies.

## 86 2. Podocytes. A Complex Structure and a singular membrane organization

87 Podocytes are highly specialized visceral epithelial cells constituting the glomerular filtration  
 88 barrier in the kidney, along with endothelial cells and the basement membrane. They ensure the  
 89 impermeability of the barrier to high molecular weight molecules, most of the blood proteins, by an  
 90 intricate system of interdigitated cellular prolongations (foot processes) and a particular  
 91 organization of cell junctions (slit diaphragm). Alterations in these structures lead to increased  
 92 permeability, resulting in proteinuria. This is a characteristic feature of podocytopathies, which  
 93 include IgA nephropathy, membranous nephropathy, and idiopathic nephrotic syndrome (INS).  
 94 Podocytopathies can also be secondary to other morbidities, such as diabetes (diabetic nephropathy)  
 95 and systemic lupus erythematosus (lupus nephritis).

96 Some individual features of the lipid composition of podocytes have been known for a long  
 97 time, contributing to a progressive comprehension. Though a global view of the podocyte lipidome  
 98 is still lacking, in spite of the information provided by a few lipidomic studies [6-8]. Cholesterol was  
 99 early revealed as a key component of podocyte membrane. Initially found in normal rat podocytes

100 as especially present in the urinary side and less abundantly in the basal side [9], it was shown as a  
101 key component of the slit diaphragm through its interaction with podocin [10], one of the resident  
102 proteins in this raft-like structure [11].

103 Another slit-diaphragm protein, nephrin, was found associated with lipid rafts, and this  
104 association necessary for nephrin phosphorylation, a modification related to slit diaphragm integrity  
105 [12]. Its presence in rafts, defined as TritonX-100 insoluble microdomains, was found partial,  
106 meaning that only one pool of cellular nephrin is associated with these structures. That pool was  
107 found to interact with podocin and the actin cytoskeleton via the CD2-associated protein (CD2AP)  
108 adaptor [13, 14]. Mutations in nephrin and podocin genes, which are at the origin of genetic  
109 nephrotic syndrome, are known to abolish lipid raft localization [15, 16], highlighting the relevance  
110 of these microdomains to podocyte function. Lipid rafts in the podocyte have also been proven  
111 involved in the regulation of transient receptor potential canonical type 6 (TRPC6) channels [17],  
112 which play a key role in the regulation of podocyte morphology and the pathogenesis of INS. The  
113 ensemble of these reports indicate that podocyte lipid rafts and slit diaphragm correspond to very  
114 close structures and display a tight functional association, with raft resident proteins contributing to  
115 the slit diaphragm spatial organization [18]. The role of sphingolipids in the podocyte has been  
116 extensively studied and reviewed by Fornoni and colls. [19].

### 117 3. Gangliosides in podocytes

118 In spite of the extreme structural diversity of gangliosides, only a handful of them have been  
119 identified in podocytes and their functions outlined in physiologic and pathologic conditions. A  
120 pioneering biochemical and immunohistochemical work mapped several gangliosides in different  
121 segments of kidneys from different species, also comparing the developing and the mature organ  
122 [20]. In the glomerulus, a transition from more complex to less complex gangliosides was observed  
123 along organ maturation. A global view of the ganglioside content within the sphingolipid landscape  
124 in podocytes has been provided by comprehensive profiling on immortalized human podocytes by a  
125 combination of high performance thin layer chromatography (HPTLC), radioactive labeling of  
126 sphingosine and mass spectrometry [8]. A significant majority of the identified sphingolipids  
127 correspond to neutral forms, encompassing sphingomyelin, ceramide, GlcCer, LacCer,  
128 globotrihexosylceramide, gangliotetraosylceramide, and globopentaosylceramide. GM3 accounted  
129 for 5% of total podocyte sphingolipids, while GM1b and GD1 $\alpha$  were also identified. This can be  
130 considered as a reference sphingolipid profiling of human podocytes. Nevertheless, immortalized  
131 cells must always be considered as a model in which molecular expression and distribution could  
132 differ from in vivo conditions. Also, the sphingolipid profile of human cells can differ from that of  
133 animal models, as it will be seen below.

#### 134 3.1. *O*-acetylated GD3

135 Interrogations on the biological relevance of podocyte gangliosides emanate from three  
136 independent studies. The pioneering work detecting a ganglioside as a main component of  
137 podocytes consisted of the identification of the antigen recognized by a monoclonal antibody  
138 specific of glomerular podocytes in rat kidney [21]. This antigen was characterized by ion exchange,  
139 thin layer and gas-liquid chromatography as *O*-acetylated-GD3. Subsequently, the same antibody  
140 was used to isolate podocyte rafts [12]. Acetylation of the sialic acid moiety can have profound  
141 consequences in ganglioside function. Another monoclonal antibody had been previously found to  
142 stain renal cortex and the recognized antigen characterized as a glycolipid migrating between GM2  
143 and GM1 in thin layer chromatograms [22]. From this point on, *O*-acetylated GD3 has become a  
144 marker of podocytes and used to characterize and validate podocyte cell lines [23]. GD3  
145 *O*-acetylation has been related to viral infection and to the resistance to apoptosis of tumor cells [24].  
146 Also, 9-*O*-acetyl-GD3 has been found specifically increased in rat kidney in response to lead  
147 exposure, which produces microalbuminuria [25].

148 Injection of puromycin aminonucleoside in rats induces a glomerulopathy known as  
149 puromycin aminonucleoside nephrosis (PAN). This is considered as an in vivo model of the human

150 INS form minimal change nephrotic syndrome (MCNS). The group that developed the monoclonal  
151 antibody to detect O-acetylated GD3 studied its expression in the kidney of proteinuric PAN rats.  
152 They found a significant decrease of this antigen and its precursor GD3, suggesting impaired GD3  
153 synthase (ST8Sia1) and O-acetyltransferase activities. These changes were observed to precede the  
154 onset of proteinuria [26]. Consistent with these results the RNA levels of GD3 synthase are  
155 decreased in the adriamycin injection model of focal and segmental glomerulosclerosis (FSGS, one of  
156 the INS forms) in mice [27]. Neither GD3 nor O-acetylated GD3 have been identified in human  
157 podocytes [8], which indirectly challenges both the relevance of rodent models and that of  
158 immortalized cell models in reproducing disease features. Results of PAN stimulation are in any  
159 case extremely important to underline the implication of gangliosides in the regulation of podocyte  
160 function, and points at O-acetylation as a modification that could impact profoundly ganglioside  
161 function.

### 162 3.2. GM3 and GD3

163 To date, one of the most relevant and comprehensive functional studies on podocyte  
164 gangliosides was an elegant work by Jin and colls. [6]. The beauty of their finding is the fact that  
165 gangliosides were indirectly identified as extremely important in podocyte biology. They used  
166 immunoprecipitation to find the interactors of the soluble vascular endothelial growth factor (VEGF)  
167 receptor 1 (Flt1) on the surface of podocytes, resulting in the unbiased detection by mass  
168 spectrometry of gangliosides, and further GM3, as the main interactor. Additional functional studies  
169 linked this interaction with cytoskeleton reorganization, which is a cornerstone in the function of the  
170 glomerular barrier.

171 Immunohistochemical and electron microscopy analysis of human kidney specimens have  
172 confirmed the specific presence of GM3 in the foot processes of podocytes [28], comforting the  
173 functional data described in the previous paragraph [6]. This confirms the high abundance of this  
174 type of ganglioside in podocytes, and its participation in maintaining the functional integrity of the  
175 filtration barrier. As a negatively charged molecule, it contributes to the protein-impermeability of  
176 the structure. This implies that any alterations in the metabolic pathways leading to decreases in  
177 GM3 might result in altered foot processes and in permeability to proteins.

178 Another unbiased study was performed by our team on mouse podocytes transfected with a  
179 vector encoding the protein CMIP (c-maf inducing protein), a podocyte and lymphocyte marker of  
180 INS [29]. Untargeted differential lipidomics of CMIP-expressing and non-expressing cells resulted,  
181 surprisingly, in a downregulation of the most abundant gangliosides (GM3, GM2 and GD2), while  
182 GM3 was identified as the most abundant podocyte component of the ganglio series. Interestingly,  
183 GM1 was not decreased [7]. The results suggested that an alteration in the pathway leading to  
184 ganglioside biosynthesis could play a role in the pathogenesis of INS. Once more, it places GM3 as a  
185 main actor in podocyte and glomerular function. However, a previous study on the mouse  
186 Adriamycin model of nephrotic syndrome, the RNA levels of GM3 synthase levels were unchanged  
187 [27].

188 It has been proposed that GM3 regulates by direct interaction both the insulin and the  
189 epidermal growth factor (EGF) receptors [30-32] [33]. In diabetes-associated nephropathy (DN)  
190 glomerular hypertrophy and proteinuria are observed. While the levels of circulating gangliosides  
191 have been correlated with albuminuria in DN patients [34], GM3 has been found increased in kidney  
192 and other tissues, along with glucocerebroside, in a streptozotocin-induced model of diabetes [35,  
193 36]. Therefore, GM3 appears as an actor of podocyte injury in DN. However, another study on the  
194 same diabetic rat model, several gangliosides, mainly GM3, were found decreased in glomeruli, in  
195 parallel to a reduction in sialic acid content [37]. A more recent work compared the tubular and  
196 glomerular content in GM3 in rat models of type 1 and type 2 diabetes. GM3 was found increased in  
197 renal tubules in both models as compared to controls, but glomeruli showed a weak increase in type  
198 1 and no change in type 2 diabetes [38]. In all these works, GM3 was semi-quantified by thin layer  
199 chromatography and the tissue localization determined by immunofluorescence with a monoclonal  
200 antibody. Using mass spectrometry imaging, glomerular and tubular ganglioside levels have been

201 reported as increased, along with other lipid classes, in a DN mouse model [39]. The development of  
202 lipidomics should provide a final answer to the intriguing dynamics of GM3 profile associated with  
203 diabetes [3, 30].

204 GM3 levels can be regulated either by ST3GAL5 or by the degrading enzyme neuraminidase 3  
205 (NEU3), or by both. In the form of nephritis developed by more than a half of patients with lupus  
206 erythematosus, an increased presence of hexosylceramides and LacCer (the ganglioside immediate  
207 precursors), and a decreased NEU sialidase activity has been reported in the kidney and urine of  
208 these patients [40]. Although increased NEU activity should be associated with decreased GM3  
209 levels, the latter were unexpectedly increased in a mouse model of lupus nephritis [41], suggesting a  
210 complex imbalance between synthesis and catabolism of gangliosides.

211 A question inferred from these studies is whether experimental blocking of ganglioside  
212 biosynthesis impacts renal function. In this line, ST3GAL5 knockout mice have been developed and  
213 have provided capital information. For instance, insulin signaling is enhanced upon GM3 synthase  
214 invalidation [42]. However, to date no renal phenotype has been described in this model. A  
215 podocyte specific inducible model of ST3GAL5 invalidation could help complete the picture of GM3  
216 function in glomeruli.

217 Interestingly, alpha-galactosidase A activity has been found significantly decreased in blood  
218 specimens from FSGS patients in hemodialysis as compared to non-FSGS control patients [43]. This  
219 enzyme is mutated in Fabry disease, in which lysosomal accumulation of globotriaosylceramide has  
220 been reported. LacCer is also the precursor of the lacto and globo series (Figure 2), and an impaired  
221 flux to GM3 and other gangliosides could be associated -as a cause or a consequence- with a  
222 detoured metabolism to globosides in FSGS. The data available to date are insufficient to establish a  
223 concrete hypothesis and a thorough analysis of these pathways in FSGS should be performed.

### 224 3.3. GM2

225 Mutations in the genes encoding the enzymes involved in glycosphingolipid metabolism result  
226 in lysosome storage disorders [44], characterized by accumulation of specific lipids depending on  
227 the metabolic reaction that is blocked by the mutation. For example, Sandhoff disease, due to  
228 hexosaminidase deficiency, leads to accumulation of GM2, since hexosaminidase is responsible for  
229 removal of N-acetylgalactosamine (GalNAc) from GM2 and subsequent retroconversion to GM3.  
230 This was first detected in brain and hepatic tissues from a patient, where lipid analysis of the kidney  
231 revealed an accumulation of globoside [45]. In Fabry disease, the accumulation of  
232 globotriaosylceramide leads to proteinuria and podocyte injury [46].

233 Mesangial cells, another cell component of glomeruli, undergo hypertrophy and proliferation  
234 in DN. This is induced *in vivo* by glucosamine administration, which results in increased levels of  
235 GM1 and GM2. Exogenous administration of these two molecules also leads to the same effects and  
236 point to GM2 accumulation by hexosaminidase deficiency as a mechanism of glomerular alteration  
237 in DN [47].

### 238 3.4. GA1

239 LacCer is not only the precursor of GM3 and, subsequently of a and b-series gangliosides, but  
240 also a particular class of glycosphingolipids known as asialo-gangliosides or 0-series gangliosides  
241 (Figure 2). This includes GA2 and GA1, respectively containing one and two galactose residues in  
242 addition to glucose and GalNAc, but exceptionally no sialic acid. In the context of DN, an  
243 untargeted, unbiased metabolomic study has recently pointed at GA1 as a circulating marker of  
244 renal function [48]. In the study, diabetic patients were subdivided into three groups according to  
245 their estimated glomerular filtration rate (eGFR), whose low levels are characteristic of renal  
246 dysfunction. The results identified plasma levels of GA1 as negatively correlated with eGFR,  
247 suggesting it as a precocious marker of kidney damage and risk of end stage renal disease. This is to  
248 our knowledge the only work showing a ganglioside as a circulating biomarker of renal disease. It is  
249 difficult to associate this finding with the above described alterations in ganglioside profile in  
250 diabetic glomeruli. It could be a sign of either a block in alpha-2,3-sialyltransferase (ST3GAL1),

251 which would be accompanied by accumulation of GM1a, GD1b, GT1c, or by increased desialylation  
252 of GM1b due to NEU1/4 activity. It could also be a sign of increased flux towards ganglioside  
253 synthesis.

254

#### 255 4. Lessons from APOL1 genetic variants

256 A polymorphism in the gene encoding apolipoprotein L1 (APOL1) has been associated with the  
257 development of several nephropathies, including hypertension-attributed nephropathy, and  
258 glomerulopathies like HIV-associated nephropathy (HIVAN) and idiopathic FSGS. The pathogenic  
259 mechanism linking the variant APOL1 alleles with podocyte damage concerns lysosomal and  
260 mitochondrial function, as well as autophagic flux [49, 50]. An exhaustive study inquired about the  
261 potential effect of APOL1 pathologic allelic variants (known as G1 and G2) on sphingolipid  
262 metabolism [8]. Transfection of human podocytes with wild-type APOL1 induced profound changes  
263 in the sphingolipid profile, in particular a dramatic decrease in LacCer content, with no changes in  
264 gangliosides. Conversely, the APOL1 allele variants induced a significant increase in GD1 $\alpha$  and  
265 sphingomyelin, and a decrease in GA1, ceramide, GlcCer, in addition to LacCer. No changes were  
266 observed in the most abundant gangliosides, such as GM3 and GM1. Strikingly, both variants and  
267 the WT form induced significant decreases in the enzymatic activities of hydrolases, such as  $\alpha$  and  $\beta$   
268 galactosidases,  $\beta$ -hexosaminidase and glucosylceramidase, responsible for the catabolic reversed  
269 reactions on the same series. But, conversely, when the authors studied these activities at the cell  
270 surface, they found the opposite effect, with increased activity, especially when the G2 variant was  
271 overexpressed. The sphingolipid profile of lipid rafts was also changed, with significant decreases in  
272 gangliosides GD1 $\alpha$ , GM3, GM1b and GA1, along with Cer, GlcCer and LacCer precursors, the  
273 changes varying depending on the APOL1 variant expressed. This suggests a role of APOL1, and  
274 possibly high density lipoproteins (HDL), in the regulation of ganglioside synthesis and  
275 degradation, and also that changes induced in sphingolipid content by genetic variants G1 and G2  
276 can contribute to podocyte alterations. But, most interestingly, changes in ganglioside species  
277 operate and are detectable especially at raft microdomains.

#### 278 5. A word by ceramides

279 Ceramides are the central metabolites where pathways converge and diverge in the  
280 sphingolipid metabolic sphere. Therefore, regulation of ceramide content will show an impact on  
281 ganglioside synthesis and, conversely, regulation of ganglioside synthesis and catabolism can have  
282 an effect on ceramide content. Ceramides themselves are bioactive lipids, but so are mostly their  
283 phosphorylated derivatives ceramide 1 phosphate (C1P) and sphingosine 1 phosphate (S1P).  
284 Ceramide levels are, therefore, tightly regulated in cells.

285 In a recent work, Li and colls. [51] characterized a mouse model in which the catalytic subunit  
286 of lysosomal acid ceramidase (Asah1) was invalidated specifically in podocytes. This enzyme is  
287 responsible for ceramide catabolism and critical in cellular homeostasis. As expected ceramides, in  
288 particular C16, accumulated in the glomeruli of these individuals. Interestingly mice developed  
289 proteinuria in the absence of morphological changes in glomeruli following light microscopy  
290 observation. Conversely, electron microscopy revealed foot process effacement. Altogether, these  
291 alterations are similar to those corresponding to MCNS. A double invalidation of Asah1 and an acid  
292 sphingomyelinase (SMPD1), an enzyme leading to ceramide production by sphingomyelin  
293 hydrolysis, corrected the ceramide levels and partially reversed the proteinuria and morphological  
294 changes observed in the single knockout. This points at ceramides as potential actors participating  
295 in the pathogenic mechanisms of podocytopathies. Also, the fact that these enzymes are lysosomal  
296 underlines the importance of these organelles in sphingolipid and glycosphingolipid homeostasis.  
297 No information is provided in the study about the status of the ganglioside flux.

298 In our works we have found, along with a decrease in GM3, GM2, and GD3 in mouse podocytes  
299 transfected with human CMIP, an increased presence of LacCer, GlcCer and several ceramides,



300 consistent with a block in GM3 synthesis at the ST3Gal5/GM3 synthase level [7]. Our finding in  
301 mouse T cells overexpressing human CMIP, consisting of a decrease in the GM3 protein expression  
302 after 30min of T-cell receptor (TCR) activation, seems to comfort the hypothesis of a GM3 synthesis  
303 blocking associated with CMIP overexpression. Interestingly, CMIP was first identified by  
304 subtractive cloning in T cells [29]. Later it was also detected in podocytes from MCNS patients [52],  
305 and the lesions induced in a mouse podocyte-specific transgenic model of CMIP overexpression are  
306 similar to those of human MCNS [52]. Considering our results in view of the findings by Li and colls.  
307 [51], it is tempting to hypothesize that ceramide participates in CMIP-induced proteinuria due to  
308 ceramide accumulation in podocytes as a consequence of the GM3 synthesis blocking.

309 A nephropathy associated with ceramide accumulation is one of the features of Farber disease,  
310 a genetic disorder resulting from mutations in the *ASAH1* gene [53]. Ceramide levels are also  
311 increased in genetic steroid resistant nephrotic syndrome in association with mutated S1P lyase  
312 [54-56]. Likewise, pharmacological inhibition of this enzyme induces a nephrotic syndrome in  
313 rodents [57]. The nephropathy associated with diabetes also benefits from the targeting of ceramide  
314 accumulation by an adiponectin receptor agonist [58]. Acid sphingomyelinase overexpression also  
315 leads to ceramide accumulation and to glomerular sclerosis in mice [59]. All these reports point at  
316 ceramide accumulation as a contributor to podocyte and glomerular dysfunction, as a result of  
317 defects in different genes and proteins. Likewise, a block in ganglioside synthesis could represent an  
318 additional source of ceramide accumulation leading to similar consequences and place ceramides at  
319 the central point of podocyte injury.

320 However, the latter hypothesis is challenged by the seminal works by Fornoni and collaborators  
321 on the connection between sphingomyelinase-like phosphodiesterase 3b (SMPDL3b) and podocyte  
322 dysfunction. While SMPDL3b is overexpressed in diabetic nephropathy patients [60], recurrent  
323 FSGS patients present a decreased expression of this enzyme in podocytes [61]. This apparently  
324 contradicting scenario might indicate that these two diseases are too far apart pathogenically,  
325 though sharing the glomerular dysfunction. Nevertheless, to our knowledge no systematic ceramide  
326 analysis has been performed to date on patients' biopsies.

## 327 **6. The role of the immune system**

328 The direct implication of the immune system in podocytopathies such as IgA nephropathy,  
329 membranous nephropathy and lupus nephritis is clear, all these based on the development of  
330 autoantibodies. In the case of INS, there are no immune depots present in the glomerulus, yet an  
331 immune dysfunction at the origin of the pathology is supported by a consistent body of evidence  
332 [62]. Nonetheless the precise pathogenic mechanisms involved in INS are far from being  
333 understood. Gangliosides exert capital functions in immune cells, (for review [63, 64]). They are  
334 present in both myeloid and lymphoid cell populations, as well as in hematopoietic stem cells [64].  
335 Thus, GM3 has been described in most immune cell types, except in eosinophils, basophils and NK  
336 cells, in which GM1 and asialo GM1 have been reported and seem to represent the most abundant  
337 ganglioside species. O-acetylated forms of GD3 have also been described in T, B and NK cells. An  
338 example of the ganglioside function in the immune system is their implication in T cell activation  
339 [65-67], where distinct profiles of gangliosides are characteristic of CD4 and CD8 cells. Ganglioside  
340 function in immune cells, as in podocytes, is related to their role in organizing membrane  
341 microdomains, but also to their interaction with cellular receptors and signal transduction.  
342 Consequently, a dysregulation of ganglioside metabolism can be expected to participate in the  
343 immune origin of INS. To date, an abnormal distribution of GM1 has been observed in T cells  
344 overexpressing the INS marker CMIP, as well as a decreased GM3 synthase expression after TCR  
345 activation [7].

346 Activation of invariant NK cells by glycosphingolipid-1 (GSL-1), a bacterial  
347 monoglycosylceramide, is able to protect glomeruli and reverse the effects of adriamycin injection,  
348 an in vivo model of FSGS in mouse [27]. Most interestingly, this protection was paralleled by  
349 increased expression of GM3 synthase in the kidney, concomitant with increased levels of Bcl-2,  
350 suggesting a protective role for GM3 in the kidney by engaging an antiapoptotic mechanism [68].

351 Although the link between adriamycin and GD3 synthase expression, and that between GSL-1 and  
352 GM3 expression are not well established, the same study suggests the involvement of TGF- $\beta$  and  
353 SMAD signaling [27], and opens an interesting mechanistic field to understand the role of  
354 gangliosides in glomerular biology and in INS.

355 In systemic lupus erythematosus (SLE) patients, the presence of antibodies possessing sialidase  
356 activity [69] targeting gangliosides, and anti-ganglioside antibodies targeting asialo-GM1, GM1,  
357 GM2, GM3, GT1b, GD1b, and GD3 have been reported [70, 71]. Increased GM1 has been observed in  
358 peripheral CD4+ T cells [72]. Abnormal T cell responses in SLE have been associated with an  
359 abnormal ganglioside profile [73, 74]. Unfortunately, there is little information about the ganglioside  
360 profile in podocytes in SLE, which is limited to date to the increased presence of GM3 in kidneys  
361 from a nephritic mouse model [41]. Other renal pathologies, such as IgA nephropathy,  
362 Henoch-Schönlein purpura nephritis, MCNS, mesangial proliferative glomerulonephritis and  
363 membranoproliferative glomerulonephritis have been associated with the presence of antibodies  
364 against N-glycolyl GM3, a variant of GM3 characteristic of some cancer cells [75]. A case of  
365 hyperthyroidism accompanied by hematuria, proteinuria, proliferation of mesangial cells and  
366 increased mesangial matrix with focal segmental capillary wall abnormality, was attributed to the  
367 presence of a thyroid antibody targeting fucosyl-GM1. The latter was detected by  
368 immunofluorescence in the glomerular basement membrane [76]. The role of anti-ganglioside  
369 antibodies in the pathogenesis of these diseases is still unexplored.

## 370 7. A call for deep analysis

371 All the observations described above, the subsequent hypotheses and insights, the potential  
372 pathogenic mechanisms, will benefit from further research based on state-of-the-art analytical  
373 strategies. It must be clarified that the reported gangliosides in the works published so far (i.e. GM3)  
374 do not correspond to single molecular entities, but to groups of molecules sharing a particular  
375 poly-sugar sialic acid-containing moiety, but differing in the nature of the sphingoid base and fatty  
376 acid chains present, which complicates significantly the research from the analytical point of view.

377 Significant improvements have been made in the last ten years for the analysis of gangliosides  
378 with the advent of lipidomics technologies [77]. Notably, the presence of polar oligosaccharide  
379 moieties that include sialic acid residues linked on their ceramide backbone, makes gangliosides  
380 particularly water-soluble. Therefore, during sample preparation, gangliosides tend to partition into  
381 a more polar or aqueous layer rather than in an organic layer as observed with other lipid classes.  
382 Once extracted from their biological matrices, a low-cost procedure for the qualitative evaluation of  
383 the endogenous ganglioside pattern is represented by HPTLC. Using this technique, the  
384 gangliosides contained in the aqueous phase of the lipid extract are separated according to the  
385 different composition of their carbohydrate structure using specific solvent systems. Lipids are then  
386 visualized using different strategies such as: i) chemical detection, ii) binding assay using antibodies,  
387 iii) carbohydrate recognition reagents, and identified by co-migration with authentic lipid standard  
388 [78]. In spite that this technique gives a rapid result related to the ganglioside composition of cells,  
389 the main issue is represented by the requirement of a relative high amount of biological sample. To  
390 increase the sensitivity of this methodology, it was exploited the use of radioactive precursors of  
391 sphingolipids in tracer concentration. In particular, cells are treated with [1-<sup>3</sup>H]-sphingosine, or  
392 [3-<sup>3</sup>H]-sphingosine, or [<sup>3</sup>H]serine or [<sup>14</sup>C]serine to obtain the metabolic labelling at the steady state of  
393 all cell sphingolipids. After incubation, gangliosides isolated in the aqueous phase are separated by  
394 TLC and radioactive lipids visualized by digital autoradiography. Exploiting the use of radioactivity  
395 and the sensitivity of digital autoradiography, the amount of biological samples to be analyzed is  
396 reduced by 1/100 with respect to that of the endogenous counterpart. As a weakness, the use of  
397 radioactive precursors is mainly applicable to cells in culture.

398 Interestingly, the use of [1-<sup>3</sup>H]-sphingosine gives also information related to sphingolipid  
399 turnover. [1-<sup>3</sup>H]-sphingosine, when administered to cells, is used for the de-novo biosynthesis of  
400 sphingolipids, which become radioactive. Radioactive lipids are then degraded in lysosomes to  
401 obtain saccharides or choline, fatty acid, and [1-<sup>3</sup>H]-sphingosine. The radioactive sphingosine could


















402 be further used into the biosynthetic pathway or could be phosphorylated to obtain  
 403 [1-<sup>3</sup>H]-sphingosine-1-phosphate (S1P). S1P is further degraded to hexadecenal and  
 404 phosphoethanolamine, which is radioactive due to the presence of tritium on the first carbon of  
 405 sphingosine. [<sup>3</sup>H]phosphoethanolamine is used for the biosynthesis of radioactive  
 406 phosphatidylethanolamine. For this reason, the use of [1-<sup>3</sup>H]-sphingosine allows to obtain the  
 407 metabolic labeling of all cell sphingolipids and also of phosphatidylethanolamine, which reflects the  
 408 rate of sphingolipids and sphingosine turnover [8, 78, 79]. One of the main limitations of HPTLC  
 409 methodology is the incapability to provide quantitative data and the information related to ceramide  
 410 structures, both evaluable by mass spectrometry (MS) analyses. MS, either electrospray ionization  
 411 (ESI) or direct analysis ionization sources, such as matrix-assisted laser desorption ionization  
 412 (MALDI), are routinely used to monitor and quantify levels of gangliosides in biological samples  
 413 [80]. Since different ganglioside species may have the same molecular mass, MS is often used in  
 414 conjunction with separation techniques, such as chromatography and ion mobility, to separate  
 415 isomers and characterize the complex and diverse chemical structures of gangliosides [81]. Recently,  
 416 imaging MS has been used to facilitate ganglioside analysis in heterogeneous tissues, such as kidney  
 417 and brain samples in particular, allowing to monitor their concentration and generate images of  
 418 their molecular composition in fine anatomical structures and substructures [82].

## 419 8. Conclusions

420 In spite of the extreme structural diversity of gangliosides, only a handful of them have been  
 421 identified in podocytes and their functions in physiologic and pathologic conditions outlined (Table  
 422 1). Nevertheless, the cellular features of podocytes and the abundance of GM3 point at a relevance of  
 423 gangliosides and their potential involvement in the pathophysiology of glomerulopathies. The role  
 424 of the immune system in INS has been demonstrated, but the mechanisms are still in the dark.

425 **Table 1.** Changes in the abundance of gangliosides and precursors associated with glomerular  
 426 diseases. Arrows indicate relative abundance variations and in parenthesis the tissue, cell or  
 427 subcellular compartment where these variations have been observed.

Molecular species	Pathology	Observed changes	Reference
Ceramide	APOL1 associated FSGS	↓ (podocytes)	[8]
	Proteinuria model (Asah1 KO mouse)	↑ (lysosomes)	[51]
	Genetic steroid resistant nephrotic syndrome	↑ (glomeruli)	[54-56]
	Glomerular sclerosis (acid sphingomyelinase overexpression)	↑ (glomeruli)	[59]
GlcCer	APOL1 associated FSGS	↓ (podocytes)	[8]
LacCer	Lupus nephritis	↑ (kidney)	[40]
	APOL1 associated FSGS	↓ (podocytes)	[8]
GM3	INS (CMIP overexpression)	↓ (podocytes)	[7]

	DN (streptozotocin rat model)		(kidney)	[35, 36]
	DN (streptozotocin rat model)		(glomeruli)	[37]
	DN (type 1 diabetes rat model)		(glomeruli)	[38]
	DN (mouse model)		(glomeruli)	[39]
	APOL1 associated FSGS		(podocyte rafts)	[8]
	Lupus nephritis (mouse model)		(kidney)	[41]
	IgA nephropathy, Henoch-Schönlein purpura nephritis, MCNS, mesangial proliferative glomerulonephritis, membranoproliferative glomerulonephritis		(antibodies)	[75]
GD3	PAN nephropathy rat model		(kidney)	[26]
O-acetylated-GD3	Microalbuminuria associated with lead toxicity		(kidney)	[25]
	PAN nephropathy rat model		(kidney)	[26]
GM2	INS (CMIP overexpression)		(podocytes)	[7]
	DN (glucosamine administration)		(mesangial cells)	[47]
GD2	INS (CMIP overexpression)		(podocytes)	[7]
GM1	DN (glucosamine administration)		(mesangial cells)	[47]
	APOL1 associated FSGS		(podocyte rafts)	[8]
GD1 $\alpha$	APOL1 associated FSGS		(podocytes)	[8]
	APOL1 associated FSGS		(podocyte rafts)	[8]
GA1	DN		Neg. correlation with eGFR	[48]

APOL1 associated FSGS

  
(podocytes)

[8]

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428 As stated in chapter 3.2, the general ST3Gal5 knockout mouse model does not develop any  
429 renal phenotype [83], whereas a podocyte-specific invalidation has not been developed to date.  
430 Cross breeding of this model with others can provide capital information on the role of ganglioside  
431 homeostasis in pathophysiology. One example in the context of kidney disease is the cross breeding  
432 of ST3Gal5 knockouts with a transgenic mouse line bearing the juvenile cystic kidney mutation (jck),  
433 responsible for polycystic kidney. Breeding results in a milder polycystic pathology, suggesting that  
434 GM3 synthase is involved in the pathogenesis [84]. Similar strategies would complement analytical  
435 studies in developing consistent hypotheses on the pathophysiologic role of ganglioside synthases  
436 and gangliosides.

437 The implementation of MS-based lipidomic approaches, targeting the biochemical pathways for  
438 ganglioside biosynthesis and degradation, will allow a better understanding of the role played by  
439 these sphingolipids in kidney diseases. Clinical strategies, based on these state-of-the-art analytical  
440 approaches, might put this category of lipids as candidates for diagnostic and prognostic biomarkers  
441 of diverse forms of podocytopathies. A long path is still ahead.  
442

443 **Abbreviations**

APOL1	Apolipoprotein L1
Asah1	Lysosomal acid ceramidase
CMIP	IC-maf inducing protein
DN	Diabetes-associated nephropathy
EGF	Epidermal growth factor
eGFR	Estimated glomerular filtration rate
ESI	Electrospray ionization
FSGS	Focal and segmental glomerulosclerosis
Flt1	Vascular endothelial growth factor receptor 1
GalNAc	N-acetylgalactosamine
GlcCer	Glucosylceramide
GA	Asialo gangliosides
GD	Di-sialo gangliosides
GM	Mono-sialo gangliosides
GP	Penta-sialo gangliosides
GQ	Quadri-sialo gangliosides
GSL-1	Glycosphingolipid-1
GT	Tri-sialo gangliosides
HDL	High density lipoproteins
HPTLC	High performance thin layer chromatography
INS	Idiopathic nephrotic syndrome
LacCer	Lactosylceramide
MALDI	Matrix assisted laser desorption ionization
MCNS	Minimal change nephrotic syndrome
NEU	Neuraminidase
PAN	Puromycin aminonucleoside nephropathy
ST8Sia1	Ganglioside D3 synthase
SMPDL3b	Sphingomyelinase-like phosphodiesterase 3b
ST3Gal1	alpha-2,3-sialyltransferase
ST3Gal5	Ganglioside M3 synthase
SLE	systemic lupus erythematosus
TCR	T-cell receptor
VEGF	Vascular endothelial growth factor

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