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1 **Castrated autoimmune glomerulonephritis mouse model shows attenuated**  
2 **glomerular sclerosis with altered parietal epithelial cell phenotype**

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5 **Short Title:** Castration attenuates glomerular sclerosis in mice

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## 20 **Abstract**

21 Sex hormones help in maintaining proper immunity as well as renal homeostasis in mammals, and  
22 these multi-functional properties characterize the onset of sex-dependent diseases. To clarify the  
23 contribution of sex hormones to autoimmune disease-related renal pathogenesis, BXSB/MpJ-*Yaa* was  
24 investigated as a murine autoimmune glomerulonephritis model. BXSB/MpJ-*Yaa* and its wild-type,  
25 BXSB/MpJ-*Yaa*<sup>+</sup> were castrated or sham-operated at 3 weeks and examined until 6 months of age.  
26 Both castrated strains showed significantly lower serum testosterone levels and body weights than  
27 sham-operated mice. Castration did not change the disease phenotypes in BXSB/MpJ-*Yaa*<sup>+</sup>. At 3  
28 months, both sham-operated and castrated BXSB/MpJ-*Yaa* manifested splenomegaly, autoantibody  
29 production, and glomerulonephritis, and castrated BXSB/MpJ-*Yaa* tended to show heavier spleen  
30 weights than the sham-operated group. At 6 months, both the treated BXSB/MpJ-*Yaa* showed  
31 equivalent autoimmune disease conditions; however, castrated mice clearly showed milder glomerular  
32 sclerotic lesions than the sham-operated groups. Urinary albumin excretion in castrated BXSB/MpJ-  
33 *Yaa* was significantly milder than in sham-operated mice at 4 months, but those of both the treated  
34 BXSB/MpJ-*Yaa* were comparable at 6 months. The examined renal histopathological indices in  
35 parietal epithelial cells (PECs) were remarkably altered by castration. Briefly, castration decreased the  
36 height of PECs and total PEC number in BXSB/MpJ-*Yaa* at 6 months. For immunostaining, PECs  
37 facing the injured glomeruli of BXSB/MpJ-*Yaa* expressed CD44, an activated PEC marker, and  
38 CD44-positive PECs showed nuclear localization of the androgen receptor and proliferation marker

39 Ki67. CD44- or Ki67-positive PECs were significantly fewer in castrated group than in sham-  
40 operated BXSB/MpJ-*Yaa* at 6 months. Further, quantitative indices for CD44-positive PEC number  
41 and frequency in renal corpuscles positively correlated with glomerular sclerotic severity in  
42 BXSB/MpJ-*Yaa*. In conclusion, androgen seemed to have an effect on both systemic immunity and  
43 renal morpho-function, however, the effect on the latter could be more clearly observed in  
44 BXSB/MpJ-*Yaa*, as PEC activation resulted in glomerular sclerosis.

45

#### 46 **Keywords**

47 Autoimmune disease, Kidney disease, Glomerulonephritis, Glomerular sclerosis, Androgen

48

#### 49 **Impact Statement**

50 The prevalence of kidney disease is increasing globally. The loss of kidney function is irreversible,  
51 and dialysis and kidney transplantation are the primary treatments; however, they are complex and  
52 expensive. Importantly, most patients undergoing dialysis are men. Androgens have been suggested as  
53 a risk factor, but how androgens contribute to the pathogenesis of kidney disease remains unclear.  
54 Here, we histologically investigated the renal pathology of a glomerulonephritis model and found  
55 milder glomerular sclerotic lesions in castrated mice, suggesting an exacerbating effect of androgens  
56 on glomerular sclerosis development. Furthermore, we report a clear expression of the androgen  
57 receptor in parietal epithelial cells (PECs), but not in glomerular cells, showing altered number and

58 property owing to castration. Taken together, our results provide new evidence that androgens  
59 contribute to the pathogenesis of kidney disease by mediating the PEC phenotype; thus, it is crucial to  
60 understand the mechanism of male-dominant symptoms.

61

## 62 **Introduction**

63 In mammals, sex hormones play an essential role in maintaining proper reproductive function. In  
64 addition, sex hormones have been regarded as modulators of immune function, resulting in clear sex-  
65 related disparities in immune system diseases. Systemic lupus erythematosus (SLE), a refractory  
66 autoimmune disease showing skin rash, joint pain, and anti-dsDNA autoantibody, represents  
67 significant sex-related disparities. SLE incidence rates in pre-menopausal women to age-matched men  
68 are 8:1-15:1; however, prevalence is lower in pre-adolescent and post-menopause females.<sup>1</sup> These  
69 data suggest that estrogens are an exacerbating factor in the etiology of SLE.

70 Lupus nephritis is the most frequent complication in patients with SLE. Autoimmune factors  
71 directly affect the pathogenesis of kidney disease. Besides local inflammation in the kidney due to  
72 disrupted autoimmunity, deposition of immune complexes and complement activation lead to  
73 thickening of the glomerular basement membrane (GBM), and spike-like structures; in addition,  
74 accumulation of extracellular matrix (ECM) produced by mesangial cells and parietal epithelial cells  
75 (PECs) results in sclerotic and crescentic lesions in the glomerulus.<sup>2</sup> Importantly, studies have  
76 reported that human male patients with SLE show more severe lupus nephritis than women, whereas  
77 the prevalence of lupus nephritis in women is higher than that in men due to a higher incidence of  
78 SLE.<sup>3,4</sup> This evidence suggests the involvement of sex hormones in the pathogenesis of lupus  
79 nephritis, in association with the immune system.

80 The kidney is an organ that can be affected by sex hormones in terms of development,

81 homeostasis, and disease conditions. Female rats have been reported to have smaller kidneys than  
82 males, and castrated mice also have a decreased kidney size.<sup>5</sup> Histologically, the PECs and proximal  
83 tubular epithelial cells in mice have been found to be larger in males than in females,<sup>6</sup> suggesting the  
84 involvement of androgens in kidney function. Androgen receptor (AR) expression has been confirmed  
85 in proximal tubular epithelial cells in mice and rats.<sup>7</sup> In addition, AR mRNA expression was reported  
86 in mouse glomeruli, including podocytes, using PCR analysis, and AR proteins in cultured mesangial  
87 cells,<sup>7-9</sup> suggesting the contribution of androgens to glomerular homeostasis as well as the  
88 pathogenesis of kidney diseases. Clinical studies have reported that several kidney diseases, including  
89 glomerulonephritis, glomerulosclerosis, membranous nephropathy, and IgA nephropathy, are more  
90 prevalent and serious in males.<sup>10-12</sup> Experimental studies have supported androgens as a risk factor for  
91 kidney diseases, as shown by the protective effect of castration in several kidney disease models  
92 induced by ureteral obstruction, ischemia-reperfusion, and diabetes<sup>13-15</sup>; in contrast, estrogen is  
93 suggested to have a protective role in kidney disease, as demonstrated in human and animal models.<sup>16</sup>  
94 On the other hand, for autoimmune nephritis, we have previously reported that androgens played the  
95 protective role in pathology of autoimmune glomerulonephritis in congenic mice unlike other kidney  
96 disease models.<sup>17</sup> Furthermore, examination of autoimmune-prone NZB-derived F1 mice showed that  
97 estrogen promotes autoimmune glomerulonephritis due to excessive activation of cellular immunity.<sup>18</sup>  
98 These results suggest that autoimmune disease-related nephritis is complicatedly formed by systemic  
99 autoimmune conditions and sex-related factors.

100 The BXSB/MpJ-*Yaa* mouse (*Yaa*) is a representative model of male-dominant systemic  
101 autoimmune disease, characterized by autoantibody production and splenomegaly due to the  
102 excessive proliferation of autoreactive lymphocytes.<sup>19</sup> *Yaa* develops severe membranous proliferative  
103 glomerulonephritis (MPGN), characterized by glomerular hypertrophy, thickening of GBM, and  
104 expansion of sclerotic lesions.<sup>20</sup> *Yaa* is derived from the BXSB/MpJ-*Yaa*<sup>+</sup> (BXSB) strain, and is  
105 presumed to develop the disease due to Y-linked autoimmune acceleration (*Yaa*) mutations,  
106 duplication of some genes on the telomere region of X chromosome to that of the Y chromosome in  
107 males.<sup>21</sup> Previous studies revealed that the BXSB genome is also suspected to develop autoimmune  
108 disease-prone phenotypes, as aged female BXSB manifested autoantibody production and  
109 glomerulonephritis.<sup>22</sup> In addition to *Yaa* mutation, we have reported that podocyte injuries due to the  
110 immune-associated genes on chromosome 1 contribute to the etiology of glomerulonephritis in *Yaa*,  
111 resulting in albuminuria. The effect of gonadectomy on *Yaa* was previously reported; there was little  
112 effect on their phenotypes; nevertheless, castrated *Yaa* was slightly accelerated to develop the  
113 autoimmune disease.<sup>23</sup> However, detailed pathology of autoimmune glomerulonephritis in *Yaa*,  
114 contributed by sex hormones has not been discussed so far.

115 In the present study, we histologically investigated the influence of castration on the  
116 pathogenesis of systemic autoimmune disease and glomerulonephritis in *Yaa*. Castrated *Yaa*  
117 accelerated the development of the autoimmune disorder represented by early splenomegaly at 3  
118 months, but there was no difference in renal pathology. At 6 months, castrated *Yaa* clearly showed

119 milder sclerotic lesions in glomeruli, although autoimmune indices were equivalent between  
120 treatments. These results show that androgens mediate the onset of systemic autoimmune disease  
121 condition at an early stage but exacerbate glomerular sclerosis with priority over the effect of  
122 autoimmunity in the later stages of the disease in Yaa.

123

## 124 **Materials and methods**

### 125 *Animals and sample preparation*

126 BXSB (without *Yaa* mutation) and Yaa mice were purchased from Japan SLC, Inc. (Hamamatsu,  
127 Shizuoka, Japan). Mice were castrated or sham-operated at 3-weeks-old, under combination  
128 anesthesia (0.3 mg/kg of medetomidine, 4.0 mg/kg of midazolam, and 5.0 mg/kg of butorphanol) with  
129 buprenorphine (0.1 mg/kg). First, the middle of the scrotum was incised in both the treated groups.  
130 Testes from both sides, epididymis, and part of the deferent duct were removed in the castrated group.  
131 During sham-operation, the testes were just held and pulled lightly, and then were placed back into the  
132 scrotum. Mice were maintained according to the Guide for the Care and Use of Laboratory Animals of  
133 Hokkaido University, and all animal experiments were approved by the Institutional Animal Care and  
134 Use Committee of Hokkaido University and the Faculty of Veterinary Medicine, Hokkaido University  
135 (approval No. 15-0079, 20-0012). Our animal experiment program was approved by the Association  
136 for Assessment and Accreditation of Laboratory Animal Care International. Urine was continuously  
137 collected from mice 3 to 6 months of age, by pressure urination. At 3- or 6-months old, under deep

138 anesthesia, after body weights were measured, blood samples were collected by cutting the carotid  
139 artery. After euthanasia by cervical dislocation, the kidney and spleen were collected. The weights of  
140 the kidney and spleen were measured, and the ratio to body weight was compared in each group.

141

#### 142 ***Serological and urinary analyses***

143 Serum and urine were used to evaluate physiological parameters. Serum testosterone levels were  
144 measured using the Testosterone ELISA Kit (IBL, Gunma, Japan), to evaluate male reproductive  
145 functions. In addition, serum anti-double-stranded DNA (dsDNA) antibody levels were measured  
146 using the Mouse Anti-dsDNA ELISA Kit (Shibayagi, Gunma, Japan), to evaluate systemic  
147 autoimmune conditions. As for renal function analysis, serum blood urea nitrogen (BUN) and serum  
148 creatinine (Cre) were measured using a Fuji Dri-Chem 7000v instrument (Fujifilm, Tokyo, Japan).  
149 Urinary albumin and creatinine levels were measured using the LBIS Mouse Albumin ELISA Kit  
150 (FUJIFILM Wako Pure Chemical Corporation) and Urinary Creatinine Assay Kit (Detroit R&D, Inc.;  
151 Detroit, MI, USA), and subsequently, the urinary albumin creatinine ratio (uACR) was calculated. We  
152 followed the manufacturer's instructions for performing all the assays.

153

#### 154 ***Histological analysis***

155 The collected organs were fixed in 4% paraformaldehyde for approximately 24 h. After  
156 embedding in paraffin, kidney samples were cut into 2- $\mu$ m thick sections. The sections were

157 deparaffinized and then stained with periodic acid-Schiff-hematoxylin (PAS-H), periodic acid  
158 methenamine-silver (PAM), and Masson's trichrome (MT) stains. In histoplanimetric analysis,  
159 histological sections were converted into digital images by scanning with NanoZoomer 2.0-RS  
160 (Hamamatsu Photonics K.K., Shizuoka, Japan). Nuclear numbers in the glomerulus and glomerular  
161 area were quantified using the NDP.view2 program (Hamamatsu Photonics K.K., Shizuoka, Japan).  
162 The sclerotic lesion area of more than 20 glomeruli was measured using sections stained with MT,  
163 using a BX analyzer (Keyence, Osaka, Japan), and the sclerotic lesion fraction was calculated as the  
164 percentage of the sclerotic area to the glomerular area.

165

#### 166 ***Immunostaining***

167       Deparaffinized sections were treated for antigen retrieval in 20 mM Tris-HCl buffer (pH 9.0) at  
168 110 °C for 15 min. For immunohistochemistry, sections were soaked in methanol containing 0.3%  
169 H<sub>2</sub>O<sub>2</sub> for 20 min at room temperature. After washing and blocking with normal goat or donkey serum  
170 for 1 h at room temperature, sections were incubated with primary antibodies, listed in Table 1, at  
171 4 °C for over 16 h. After washing, the sections were incubated with secondary antibodies, listed in  
172 Table 1. For immunohistochemistry, the sections were incubated with streptavidin-horseradish  
173 peroxidase (SABPO kit; Nichirei, Tokyo, Japan) for 30 min at room temperature, and then incubated  
174 with 3,3-diaminobenzidine tetrahydrochloride-0.3% H<sub>2</sub>O<sub>2</sub> solution. Finally, the sections were  
175 counterstained with hematoxylin. For immunofluorescence, sections were incubated with Hoechst

176 33342 (1: 500; Dojindo, Kumamoto, Japan) for nuclear staining at room temperature for 15 min. All  
177 the immunofluorescence sections were observed under an All-in-One Fluorescence Microscope BZ-  
178 X710 (Keyence, Osaka, Japan), and were analyzed using a BX analyzer.

179 In histoplanimetric analysis, immunostained sections for CD44 were converted into digital  
180 images by scanning with NanoZoomer 2.0-RS. With more than 50 renal corpuscles, the total number  
181 and CD44-positive PECs were counted, and the circumference length of each Bowman's capsule was  
182 measured with the NDP.view2 program. Then, the numbers of total and CD44-positive PECs per unit  
183 length of Bowman's capsules were calculated. In addition, the number of renal corpuscles, including  
184 the CD44-positive PECs as well as the total number of renal corpuscles in a section were calculated,  
185 followed by the evaluation of CD44-positive renal corpuscle ratio. Further, in immunofluorescent  
186 sections, the number of antigens identified using monoclonal antibody Ki67 (Mki67, also known as  
187 Ki67)-positive PECs was counted by observing under a BZ-X710 microscope, in more than 100 renal  
188 corpuscles, and the number of Ki67-positive PECs in a renal corpuscle was calculated.

189

#### 190 ***Reverse transcription and quantitative PCR (qPCR)***

191 Total RNA was isolated from the kidneys using TRIzol Reagent (Life Technologies, Carlsbad,  
192 CA, USA), following the manufacturer's protocol. cDNA was synthesized from total RNA by reverse  
193 transcription (RT) using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO,  
194 Osaka, Japan). Gene expression levels were examined using synthesized cDNA, THUNDERBIRD

195 SYBR qPCR Mix (TOYOBO, Osaka, Japan), and a real-time thermal cycler (CFX Connect; BIO-  
196 RAD, California, USA), following the manufacturer's instructions. Gene expression in the testes was  
197 normalized to the expression of actin beta (*Actb*). The details of the primers are shown in Table 2.

198

### 199 ***Statistical analysis***

200 The results are expressed as mean  $\pm$  standard error (SE), and were analyzed using non-parametric  
201 statistical methods. Data between different treatments in the same strain, different strains with the  
202 same treatment, and different ages in the same strain with the same treatment were compared using  
203 the Mann-Whitney *U*-test. Spearman's correlation coefficient was used to analyze the correlation  
204 between two parameters. All the values were considered statistically significant at  $P < 0.05$ .

205

206

207

208 **Results**

209 *Physiological change in castrated mice*

210 We first compared the physiological parameters of sham-operated and castrated mice to evaluate the  
211 effect of castration. Figure 1a shows the blood testosterone level of each group. All castrated mice at  
212 both ages showed significant decrease in their testosterone levels compared with those of sham-  
213 operated mice. Body weights of all castrated mice were also lower than those of sham-operated mice  
214 of the same strain at 3 and 6 months (Fig 1b). Castration did not induce any change in the spleen  
215 weight of BXSB mice, but decreased the body weight in castrated mice at both ages (Fig 1c and d).  
216 On the other hand, castrated Yaa at 3 months showed a tendency of increased spleen weight compared  
217 to the sham-operated group ( $P = 0.083$ , Fig 1c), which resulted in a significant difference in spleen  
218 weight to body weight ratio (Fig 1d). On histological examination of the spleen, Yaa showed  
219 expanded white bulb area compared to BXSB, but there was no significant difference between the  
220 treatment groups (Supplemental Fig 1a). At 6 months, there was no significant difference in spleen  
221 weight, body weight ratio, or histology between castrated and sham-operated Yaa (Fig 1d,  
222 Supplemental Fig 1b). Serum anti-dsDNA antibody levels were higher in Yaa than in BXSB of all the  
223 examined groups, but no difference was observed between castrated and sham-operated mice of both  
224 strains at 3 and 6 months (Fig 1e). These data suggest that castration evidently did not have any effect  
225 on the immune system but might affect the onset of autoimmune disease of Yaa.

226

227 ***Histological changes in the kidney of castrated mice***

228 We next examined the histology of the kidneys in mice to investigate the effect of castration on renal  
229 pathogenesis. In BXSB, sham-operated mice did not show glomerular pathology at 3 and 6 months,  
230 and there were no significant histological changes in the glomerulus due to castration (Fig 2a). On the  
231 other hand, castrated BXSB showed a flattened morphology of PEC, while the sham-operated BXSB  
232 rendered a more cuboidal shape at both the ages. Figure 2b shows representative glomerular histology  
233 of 3-month-old Yaa. Both the treated mice showed MPGN with glomerular hypertrophy and  
234 expansion of mesangial lesions, but there were no treatment-related histological changes in the renal  
235 corpuscle except for flat PEC, as observed in castrated BXSB (Fig 2b). At 6 months, severe MPGN  
236 characterized by glomerular hypertrophy, thickened GBM, and expansion of sclerotic lesions was  
237 observed in both treatment groups; however, castrated mice showed milder expansion of sclerotic  
238 lesions compared with sham-operated mice (Fig 2c). Furthermore, some sham-operated Yaa showed  
239 more severe MPGN, characterized by global sclerotic lesions and crescent formed by stratified PECs  
240 and ECM attached to the capillary tuft (Fig 2d); however, these pathological changes were scarcely  
241 observed in castrated Yaa. Comparison of histoplanimetry between different treatment groups at 6  
242 months did not alter the area of glomerulus and the number of glomerular nuclei due to castration (Fig  
243 2e and f). On the other hand, sclerotic lesion area and its fraction of glomerulus were remarkably  
244 lower in castrated mice than in sham-operated Yaa (Fig 2g and h).

245

246 ***Functional changes in podocytes of castrated Yaa kidney***

247 We further investigated the morpho-function of podocytes of 6-month-old Yaa, since formation of  
248 sclerotic lesions is considered to be the result of loss of podocyte function. Figure 3a shows  
249 immunofluorescence of podocyte slit diaphragm molecules (nephrin and podocin) and podocyte  
250 cytoskeleton molecule (synaptopodin) in Yaa glomerulus at 6 months. In both the treated Yaa,  
251 expression of all the molecules was faint at the center of the glomerulus. On comparing the  
252 treatments, partial granular patterns were observed at the glomerular edge in the sham-operated group,  
253 but a linear positive reaction was relatively maintained in the castrated Yaa for all the markers.  
254 Quantitative PCR analysis showed significantly higher levels of podocyte functional markers in  
255 castrated mice than in sham-operated mice (Fig 3b).

256 Loss of podocyte slit diaphragm molecules results in albumin leakage into the urine. uACR was  
257 compared between sham-operated and castrated Yaa from 3 to 6 months (Fig 3c). At 3 months, uACR  
258 levels were equivalent between the two treatment groups. On the other hand, castrated mice  
259 demonstrated significantly decreased levels of uACR at 4 months compared to sham-operated mice,  
260 and this tendency continued till 5 months, but without statistical significance. Eventually, uACR was  
261 comparable between the two groups at 6 months. Serum BUN and Cre in castrated Yaa were observed  
262 to be lower than those in sham-operated mice, but without statistical significance (Fig 3d and e).

263

264 ***Analysis of androgen receptor in 6-month-old Yaa kidney***

265 Next, we examined AR in Yaa kidney to determine the role of androgen in the pathogenesis of  
266 glomerulonephritis in Yaa. In 6-month-old Yaa, kidney weight was significantly decreased by  
267 castration (sham-operated (g):  $0.168 \pm 0.010$ , castrated (g):  $0.115 \pm 0.006$ ). Figure 4a shows  
268 immunohistochemistry for AR in 6-month-old Yaa kidney. In sham-operated kidneys, a clear positive  
269 reaction was detected in the nuclei of proximal tubules in the cortex and outer stripe of the outer  
270 medulla, and their cytoplasm was slightly positive. As for the cells in the renal corpuscle,  
271 immunopositive reaction was clearly detected in the nuclei of PECs in sham-operated Yaa. Castrated  
272 mice showed similar localization of AR in the kidney, but positive reactions in the nuclei of both  
273 tubulointerstitium and PECs were relatively weak as compared with those in the sham-operated Yaa.  
274 However, on comparing the mRNA levels of *Ar* in Yaa kidney, no difference was observed between  
275 sham-operated and castrated mice (Fig 4b).

276

#### 277 *Analysis of PEC phenotype in 6-month-old Yaa kidney*

278 PECs have been demonstrated to contribute in the formation of glomerulosclerosis.<sup>24</sup> In particular,  
279 activated PECs, characterized by CD44 expression on their cell membrane, are considered to have the  
280 potential to migrate, proliferate, and produce ECM, and play a role in the pathogenesis of glomerular  
281 sclerotic lesions.<sup>25</sup> We first examined the expression of CD44 in 6-month-old Yaa kidneys, using  
282 immunohistochemistry. CD44-positive PECs were rarely observed in the renal corpuscle with  
283 uninjured glomeruli in both the treated Yaa groups (Fig 5a). On the other hand, CD44-positive

284 reaction was clearly observed in the PECs of renal corpuscles with injured glomeruli showing  
285 hypertrophy and expansion of sclerotic lesions. Particularly, PECs located in the middle (equator) part  
286 of Bowman's capsule, representing middle, between flat and cuboidal, height, frequently showed  
287 CD44-positive reactions compared to other parts of the PECs in both the groups. These PECs have  
288 been classified as intermediate PECs having proliferation and migration properties<sup>26</sup>. Sham-operated  
289 Yaa clearly showed CD44 expression on their cell membrane, particularly on the surface facing the  
290 capsular lumen, but in castrated mice, cell membrane reaction was unclear due to flat morphology of  
291 PECs. In histoplanimetical analysis, CD44-positive PEC number was significantly decreased in  
292 castrated Yaa at 6 months (Fig 5b). Furthermore, castrated mice showed a lower frequency of renal  
293 corpuscles with CD44-positive PECs (Fig 5c). Figure 5d shows immunofluorescence of CD44 and  
294 AR in sham-operated Yaa at 6 months, having glomeruli with sclerotic lesions. Many PECs were  
295 positive for CD44, and stratified PECs clearly showed positive reactions for both CD44 and AR. For  
296 the analysis of proliferating cells, Ki67 was used with immunostaining. Figure 5e shows a  
297 representative immunofluorescence for CD44 and Ki67 in Yaa kidneys showing MPGN. Ki67-  
298 positive cells were observed in the glomerulus and PECs of both the treated Yaa groups at 6 months.  
299 In sham-operated Yaa, in particular, Ki67-positive reactions were observed in the stratified PECs that  
300 showed a positive reaction for CD44, whereas stratified PECs were rarely observed in castrated Yaa.  
301 Histoplanimetical analysis showed a significant decrease in Ki67-positive PEC number in castrated  
302 Yaa (Figure 5f). Further, the total number of PECs were significantly lower in castrated kidneys (Fig

303 5g).

304 Table 3 shows correlation between sclerotic lesion and examined parameters in 6-month-old Yaa  
305 of both the treatment groups. The area of sclerotic lesion showed correlation with spleen weight, but  
306 sclerotic fraction and uACR showed no correlation with autoimmune disease indices. In addition,  
307 CD44 parameters showed weak positive correlation with spleen weight, but not with anti-dsDNA  
308 antibody level. Meanwhile, both area and fraction of sclerotic lesions were significantly and positively  
309 correlated with PEC parameters, CD44-positive PEC number, CD44-positive renal corpuscle ratio,  
310 and total PEC number. The sclerotic area, in particular, showed positive correlation with CD44-  
311 positive PEC parameters. Further, uACR was also clearly associated with CD44 expression in PECs,  
312 and CD44 parameters showed positive correlation with PEC number.

313

314 **Discussion**

315 In both healthy and autoimmune disease groups, castration decreased serum testosterone levels and  
316 body weight 3 months onwards. This would be because androgens contribute to increasing the  
317 weights of bones and muscles.<sup>27</sup> In the comparison of autoimmune indices, castration seemed to  
318 accelerate the onset of autoimmune disease, but did not have any effect on the late pathological stage  
319 of Yaa. Splenomegaly is generally observed when the immune system is disrupted in certain  
320 conditions, such as autoimmune diseases and infections.<sup>28</sup> Importantly, gonadectomy could be a  
321 trigger for autoimmune disorders. In particular, the lack of androgens caused due to castration could  
322 lead to autoimmune disorders; this has been reported in a man who underwent testicular removal  
323 followed by estrogen administration therapy, and developed SLE.<sup>29</sup> The lack of immunosuppressive  
324 function of androgen could result in excessive activation of immune cell proliferation in Yaa as well.  
325 However, there was no influence of castration on BXSB immunological condition during the  
326 examination period, and there was no treatment-related difference in autoantibody levels in both the  
327 BXSB and the Yaa. Thus, male hormones could be important in suppressing autoimmunity,  
328 particularly, cellular immunity, but their absence would not affect the immune system if it is  
329 functioning normally in this experimental model.

330 Yaa represents lupus nephritis-like glomerulonephritis with the progression of systemic  
331 autoimmune disease. At 3 months, Yaa showed MPGN, but there was no treatment-related difference  
332 in renal pathology except for PEC flattening, suggesting that androgen would not be involved in the

333 onset of Yaa glomerulonephritis, although castration significantly decreased kidney weight.  
334 Importantly, female BXSB also developed splenomegaly and MPGN 10 months onwards<sup>22</sup>, while no  
335 renal lesion was observed in male BXSB without *Yaa* mutation at the same age. The present study  
336 adds evidence that androgens do not directly affect BXSB renal pathologies. These findings indicate  
337 that predispositions of the BXSB genome, including the development of MPGN, can be more  
338 sensitively affected by estrogen than androgens, even if there is a subsequent effect of androgens.

339 More severe glomerular pathology characterized by the expansion of sclerotic lesions in the  
340 glomerulus in 6-month-old Yaa would be associated with autoimmune disease progression, as shown  
341 by a positive correlation with spleen weight. On the other hand, castrated Yaa clearly showed milder  
342 sclerotic lesions compared with the sham-operated group, whereas there were no treatment differences  
343 in nuclear number and glomerular area. These results strongly suggest that formation of sclerotic  
344 lesions would be specifically affected by androgens in Yaa, even though androgen was suggested to  
345 have a protective effect in other autoimmune nephritis models. Several studies have reported that  
346 androgens promote the formation of sclerotic lesions in the glomerulosclerosis disease model<sup>15</sup>. The  
347 etiology has not been elucidated, but increase in transforming growth factor- $\beta$  level in the glomerulus  
348 by androgens could be a potent pathogenesis of glomerular sclerotic lesions<sup>9</sup>. Based on these results,  
349 autoimmune nephritis in Yaa would be complicatedly formed by genetic factors, systemic  
350 autoimmunity, and sex hormones, but the effect of androgens would be superior to  
351 immunosuppression.

352           Formation of glomerular sclerotic lesions is commonly observed in various kidney diseases,  
353 including focal segmental glomerulosclerosis, diabetes nephritis, and lupus nephritis, characterized by  
354 ECM accumulations.<sup>30</sup> Disruption of the glomerular filtration barrier that podocytes maintain is the  
355 primary pathological event, which results in the leakage of circulating proteins into primitive urine.  
356 Previous studies reported that mouse podocytes and mesangial cells express AR, and androgens have  
357 been suggested to induce apoptosis of cultured podocytes and promote ECM production in mesangial  
358 cells *in vivo*<sup>7-9</sup>. However, in the current model, AR was observed significantly in the nuclei of PECs,  
359 but not in those of podocytes and mesangial cells. Importantly, PEC is suggested to be involved in the  
360 formation of glomerular sclerotic lesions once podocytes are injured, although the molecular  
361 mechanisms are still poorly defined.<sup>24</sup> PECs tend to proliferate, produce ECM, and migrate to tuft to  
362 seal the disrupted filtration barrier. As a result, glomeruli increase sclerotic lesions due to the  
363 accumulation of ECM that PECs and mesangial cells mainly produce.

364           Recently, PECs have been divided into subpopulations classified by specific molecules,<sup>26</sup> and  
365 CD44 has been established as a marker of activated PECs.<sup>31</sup> CD44 is a cell membrane protein, which  
366 functions as a receptor of hyaluronic acid and ECM, and has been reported to be involved in cell  
367 proliferation, migration, and malignancy in various cancers, such as prostate cancer and breast  
368 cancer.<sup>32</sup> In the kidney, CD44-positive PECs are considered to have the ability to proliferate, migrate,  
369 and produce ECM as well. Furthermore, previous studies have reported the contribution of CD44-  
370 positive PECs in the formation of glomerulosclerosis and crescents.<sup>25</sup> Our results confirmed CD44

371 expression on PECs facing injured glomeruli, but not in renal corpuscles with uninjured glomeruli,  
372 and a positive correlation with sclerotic indices indicated CD44 function in the formation of sclerotic  
373 lesions in Yaa. Additionally, the decreased number of CD44-positive PECs and the positive ratio of  
374 CD44 in the renal corpuscles of 6-month-old castrated Yaa highlighted the effect of androgens on  
375 CD44 expression. The decreased number of Ki67-positive PECs suggests that the proliferation of  
376 PEC is suppressed in castrated Yaa, which might result in the decrease of total number of PECs. Thus,  
377 we infer that morpho-functional changes in PECs under androgen-poor conditions play a key role in  
378 the attenuation of glomerular sclerosis in Yaa.

379 AR is stably expressed in the cytoplasm of widespread cells and tissues. After androgens bind to  
380 AR, androgen/AR (A/AR) complexes translocate to the nucleus and function as transcription factors.<sup>33</sup>  
381 The involvement of A/AR signaling in CD44 expression has been suggested in some epithelial  
382 tumors. Prostate cancer develops androgen-dependence, and *in vitro* analysis showed that AR  
383 expression mediates CD44 expression in prostate cancer cells.<sup>34,35</sup> More importantly, androgen-  
384 mediated CD44 expression is reported to lead epithelial-mesenchymal transformation (EMT) in  
385 cancer cells, which allows cells to proliferate and produce ECM excessively.<sup>36</sup> In renal pathology,  
386 PECs are observed to undergo EMT, resulting in the proliferation and production of ECM, thereby  
387 forming crescent and glomerular sclerotic lesion.<sup>37</sup> In this study, we revealed that A/AR might mediate  
388 the PEC phenotype, in particular, expressing CD44 and undergoing EMT in Yaa glomerulonephritis.  
389 Moreover, decreased immunopositive reaction of AR in the PEC nucleus in castrated mice compared

390 with sham-operated PEC, despite equivalent mRNA expression levels of *Ar* in the whole kidney,  
391 supported the decreased activity of A/AR signaling pathway, owing to castration.

392 Castrated Yaa showed decreased levels of uACR compared to the sham group at 4 months, when  
393 MPGN progressed severely in Yaa. This suggests that castration prevents progression of renal lesions  
394 in Yaa. There is a less possibility of direct contribution of androgens to glomerular injury because  
395 there is no expression of AR in the glomerulus. It is rather reasonable that androgens had already  
396 affected PECs at 4 months to promote the formation of sclerotic lesions. Furthermore, androgen is  
397 thought to increase albuminuria in some strains of mice and rats, as male animals show higher urinary  
398 protein levels than females.<sup>38,39</sup> Albuminuria from glomerulus is reported to induce CD44 expression  
399 in PECs in a glomerulonephritis mouse model.<sup>40</sup> Positive correlation between CD44-related indices  
400 and uACR level in 6-month-old Yaa indicates their close association with Yaa as well. Higher uACR  
401 levels in sham-operated Yaa than in the castrated group at 4 and 5 months suggest that androgens  
402 induce the leakage of albumin, which could enhance CD44 expression in PECs. As a result, formation  
403 of glomerular sclerosis lesions synergistically exacerbated renal pathology in Yaa. However, at the  
404 late disease stage, castrated Yaa showed equivalent levels of uACR with sham-operated Yaa. It could  
405 be reasoned that castrated Yaa developed more severe autoimmune diseases and MPGN than in early  
406 stages, and the formation of global sclerotic lesions in sham-operated Yaa lead to glomerular  
407 dysfunction, resulting in decreased uACR level.

408 In conclusion, castration caused accelerated onset of autoimmune diseases, but attenuated

409 sclerotic lesions in Yaa, a male-dominant autoimmune disease mouse model. In the pathogenesis of  
410 autoimmune disease, androgen suppresses excessive autoimmune reactions expressed earlier during  
411 the onset of disease in castrated Yaa. In contrast, renal pathology is definitely exacerbated with the  
412 expansion of sclerotic lesions in the presence of androgen. Our results suggest the importance of the  
413 effect of androgen on the formation of glomerular sclerosis over the immunosuppressive effect in this  
414 autoimmune glomerulonephritis model. Further, PECs are suggested to be mediated by A/AR  
415 signaling in the pathogenesis of glomerular sclerosis in Yaa, which plays a critical role in this  
416 pathogenesis by proliferating and producing ECM. These findings provide novel insights into the sex-  
417 related disparity of renal diseases in zoobiquitous medicine.

418

#### 419 **Authors' Contributions**

420 Y.O., O.I., T.N., and Y.K. designed the work; Y.O., O.I., M.A., and T.N. performed experiments and  
421 analyzed the data; Y.O., O.I., and Y.K. drafted and revised the manuscript. All the authors were  
422 involved in the writing of the paper and approval of the final manuscript.

423

#### 424 **Declaration of Conflicting Interests**

425 The authors declared no potential conflicts of interest with respect to the research, authorship, and/or  
426 publication of this article.

427

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433

434 **Figure legends**

435 **Figure 1. Indices of male reproductive function and autoimmune disease condition in mice**

436 (a) The serum level of testosterone. (b) Body weight. (c) Spleen weight. (d) The ratio of spleen weight  
437 to body weight. (e) The serum level of anti-double stranded DNA antibody. (f) Kidney weight. Each  
438 bar represents mean  $\pm$  SE (n = 4-10). s: sham-operated; c: castrated. \*: Significant differences in  
439 castrated groups against sham-operated group in the same strain at the same age (\*:  $P < 0.05$ , \*\*:  $P <$   
440  $0.01$ , Mann-Whitney  $U$ -test). †: Significant differences in Yaa against BXSB with the same treatment  
441 at the same age (†  $P < 0.05$ , ††  $P < 0.01$ , Mann-Whitney  $U$ -test). #: Significant differences in 6-  
442 month-old groups against 3-month-old groups in the same strain with the same treatment (#:  $P < 0.05$ ,  
443 ##:  $P < 0.01$ , Mann-Whitney  $U$ -test).

444

445 **Figure 2. Histopathological analysis of glomeruli in mice**

446 (a) Histology of glomeruli in sham-operated and castrated BXSB at 3 and 6 months. Sections are  
447 stained with periodic acid-Schiff -hematoxylin (PAS-H). (b and c) Histology of glomeruli in sham-  
448 operated and castrated Yaa at 3 months (b) and 6 months (c). Sections are stained with PAS-H,  
449 periodic acid methenamine-silver (PAM), and Masson's trichrome (MT). At 3 months, glomerular  
450 hypertrophy, increase in mesangial matrix lesion, and wrinkling of the glomerular basement  
451 membrane were observed, but there were no treatment-related differences. These histological changes  
452 are clearer at 6 months compared with 3 months in both the treatment groups. Expansion of sclerotic

453 lesion is more significant in sham-operated Yaa than in castrated Yaa. (d) Representative histology of  
454 glomeruli with global sclerotic lesions stained with PAS-H and PAM in sham-operated Yaa at 6  
455 months. Increased parietal epithelial cells and accumulation of extracellular matrix are significant,  
456 resulting in the adhesion of glomerular tuft and Bowman's capsule. Bars = 50  $\mu$ m. (e) The area of  
457 glomeruli in sham-operated Yaa (sYaa) and castrated Yaa (cYaa) at 6 months. (f) The number of nuclei  
458 per unit area of a glomerulus in sYaa and cYaa at 6 months. (g) The area of sclerotic lesion in  
459 glomeruli in sYaa and cYaa at 6 months. (h) The fraction of sclerotic lesions in sYaa and cYaa at 6  
460 months. Each bar represents mean  $\pm$  SE (n = 8-10). Significant differences in castrated groups  
461 compared to the sham-operated group are indicated with an asterisk. \*:  $P < 0.05$ , \*\*:  $P < 0.01$  (Mann-  
462 Whitney *U*-test).

463

### 464 **Figure 3. Analysis of podocyte function in 6-month-old Yaa**

465 (a) Immunofluorescence of podocyte function molecules (Podocin, Nephlin, Synaptopodin) in Yaa at  
466 6 months. Nephlin-, Podocin- and Synaptopodin-immunopositive areas are faint at the center of the  
467 glomerulus of Yaa in both the treatment groups, but linear positive reactions at the peripheral area of  
468 glomeruli are partially eliminated in only sham-operated groups. Bars = 50  $\mu$ m. (b) Relative mRNA  
469 expression of podocyte function molecules in kidneys of sham-operated Yaa (sYaa) and castrated Yaa  
470 (cYaa). The expression levels were normalized to the levels of *Actb*. (c) uACR in sYaa and cYaa from  
471 3 to 6 months. (d and e) The serum levels of serum blood urea nitrogen (BUN, d) and serum

472 creatinine (Cre, e) in sYaa and cYaa at 6 months. Each bar represents mean  $\pm$  SE (n = 8-10).

473 Significant differences in castrated groups compared to the sham-operated group are indicated with an  
474 asterisk. \*:  $P < 0.05$ , \*\*:  $P < 0.01$  (Mann-Whitney *U*-test).

475

#### 476 **Figure 4. Analysis of androgen receptor in 6-month-old Yaa**

477 (a) Immunohistochemistry of androgen receptor (AR) in outer medulla, cortex, and glomerulus of Yaa  
478 kidneys at 6 months. Positive reaction is detected in nucleus of proximal tubules in medulla and  
479 cortex, and parietal endothelial cells (PECs). Dotted line represents the boundary between outer stripe  
480 (OS) and inner stripe (IS) in outer medulla. Arrowheads represent AR-positive PECs. Both the  
481 treatment groups show same localization, but reaction strengths in PECs tend to be lower in castrated  
482 Yaa. Bars = 100  $\mu$ m. (b) Relative mRNA expression of *Ar* in kidneys of sham-operated Yaa (sYaa)  
483 and castrated Yaa (cYaa). The expression levels were normalized to the levels of *Actb*. Each bar  
484 represents mean  $\pm$  SE (n = 8-10).

485

#### 486 **Figure 5. Histopathological analysis of parietal epithelial cells in 6-month-old Yaa**

487 (a) Immunohistochemistry of CD44 in Yaa kidneys at 6 months. Arrowheads represent CD44-positive  
488 parietal epithelial cells (PECs). In renal corpuscles with uninjured glomeruli, CD44-positive PECs are  
489 not observed in both the treatment groups. PECs in renal corpuscles with injured glomeruli show  
490 positive reaction on their cell membrane. Treatment-related change of CD44 localization is not

491 observed. Bars = 50  $\mu$ m. (b) The number of CD44-positive PECs per unit length of Bowman's capsule  
492 in sham-operated Yaa (sYaa) and castrated Yaa (cYaa) at 6 months. (c) The ratio of renal capsules with  
493 CD44-positive PECs in sYaa and cYaa at 6 months. (d) Immunofluorescence of androgen receptor  
494 (red, AR) and CD44 (green) in sYaa at 6 months. Dotted line represents renal corpuscles. Arrowheads  
495 represent both markers-positive PECs. Some of the CD44-positive PECs stratify, and some of their  
496 nuclei are positive for AR. Bar = 50  $\mu$ m. (e) Immunofluorescence of Ki67 (red) and CD44 (green) in  
497 Yaa at 6 months. Dotted line represents renal corpuscles. Arrowhead represents both markers-positive  
498 PECs. Some CD44-positive PECs stratify, and some of their nuclei are positive for Ki67 in sham-  
499 operated Yaa. Bars = 50  $\mu$ m. (f) The number of Ki67-positive PECs in a renal capsule in sYaa and  
500 cYaa at 6 months. (g) The total number of PECs per unit length of Bowman's capsule in sYaa and  
501 cYaa at 6 months. Each bar represents mean  $\pm$  SE (n = 8-10). Significant differences in castrated  
502 groups compared to the sham-operated group are indicated with an asterisk. \*:  $P < 0.05$ , \*\*:  $P < 0.01$   
503 (Mann-Whitney  $U$ -test).

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**Table 1. Antibodies**

<b>Primary Antibody</b>	<b>Source</b>	<b>Detection (Application)</b>	<b>Dilution</b>	<b>Blocking</b>	<b>Antigen retrieval</b>	<b>Secondary Antibody (IHC)</b>	<b>Secondary Antibody (IF)</b>
Rabbit anti-nephrin	IBL (Gunma, Japan)	Podocyte slit diaphragm (IF)	1:400	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-podocin	IBL (Gunma, Japan)	Podocyte slit diaphragm (IF)	1:800	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Mouse anti-synaptopodin	Fitzgerald, (MA, USA)	Podocyte cytoskeleton (IF)	1:100	5% NDS	10 mM CB (pH 6.0) 110°C, 15 min	-	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-androgen receptor	Abcam (Cambridge, UK)	Androgen receptor (IHC, IF)	1:500 (IHC) 1:200 (IF)	10% NGS (IHC) 5% NDS (IF)	20mM Tris-HCl (pH 9.0) 121°C, 10 min	Biotin-conjugated goat anti-rabbit IgG antibody (SABPO(R) Kit, Nichirei)	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rat anti-CD44	BD Biosciences (CA, USA)	Activated parietal epithelial cell (IHC, IF)	1:400 (IHC) 1:800 (IF)	10% NGS (IHC) 5% NDS (IF)	20mM Tris-HCl (pH 9.0) 110°C, 15 min	Goat anti-rat IgG antibody (CA, USA)	Alexa Fluor 488-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)
Rabbit anti-Ki67	Abcam (Cambridge, UK)	Proliferating cell (IF)	1:800	5% NDS	20mM Tris-HCl (pH 9.0) 110°C, 15 min	-	Alexa Fluor 546-labeled donkey anti-rabbit IgG antibodies (1:500; Thermo Fisher Scientific)

CB: citrate buffer. NGS: normal goat serum. NDS: normal donkey serum. IHC: immunohistochemistry. IF: immunofluorescence.

**Table 2. Primers**

Gene name (accession no.)	Official symbol	Primer sequence (5'-3')	Product size (bp)
Actin beta (NM_007393)	<i>Actb</i>	F: TGTTACCAACTGGGACGACA R: GGGGTGTTGAAGGTCTCAA	165
Nephrin 1, nephrin (NM_019459)	<i>Nphs1</i>	F: ACCTGTATGACGAGGTGGAGAG R: TCGTGAAGAGTCTCACACCAG	218
Nephrin 2, podocin (NM_130456)	<i>Nphs2</i>	F: AAGGTTGATCTCCGTCTCCAG R: TTCCATGCGGTAGTAGCAGAC	105
Synaptopodin (NM_177340.2)	<i>Synpo</i>	F: CATCGGACCTTCTCCTGTG R: TCGGAGTCTGTGGGTGAG	90

**Table 3. Correlation analysis among examined parameters in 6-month-old Yaa.**

			Autoimmune disease		Activation of PECs		
<i>Parameters</i>			Spleen weight	Serum dsDNA level	CD44-positive PEC number	CD44-positive renal corpuscle ratio	Total PEC number
<b>Sclerotic lesion</b>	Sclerotic lesion area	$\rho$	0.515*	0.003	0.863**	0.851**	0.649**
		$P$	0.029	0.991	<0.001	<0.001	0.004
	Sclerotic lesion fraction	$\rho$	0.302	-0.124	0.655**	0.638**	0.645**
		$P$	0.223	0.649	0.003	0.004	0.004
<b>Glomerular function</b>	uACR	$\rho$	0.612	0.297	0.794**	0.818**	0.527
		$P$	0.060	0.405	0.006	0.004	0.117
<b>Activation of PECs</b>	CD44-positive PEC number	$\rho$	0.417	0.196	-	0.994**	0.723**
		$P$	0.085	0.468	-	<0.001	0.001
	CD44-positive renal corpuscle ratio	$\rho$	0.475*	0.210	0.994**	-	0.719**
		$P$	0.047	0.434	<0.001	-	0.001

\* $P < 0.05$ , \*\*  $P < 0.01$ .  $\rho$ : Spearman's rank correlation coefficient, n = 18. uACR: urinary albumin creatinine ratio, PEC: parietal epithelial cell









