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Age-related glomerular lesions with albuminuria in male cotton rats

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Running headline: Glomerulus of aged cotton rats

Keywords: Cotton rats, aging, ~~male,~~ glomerulus, albuminuria, glomerular basement membrane, podocyte

Abbreviations

1. chronic kidney disease (CKD)
2. glomerular basement membrane (GBM)
3. blood urea nitrogen (BUN)
4. serum creatinine (sCr)
5. white blood cell (WBC)
6. red blood cell (RBC)
7. hemoglobin (Hgb)
8. hematocrit (HCT)
9. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)
10. urinary creatinine (uCr)
11. periodic acid Schiff (PAS)
12. periodic acid methenamine silver (PAM)
13. phosphate-buffered saline (PBS)

Abstract

The increased prevalence of aging-related chronic kidney disease (CKD) among humans is a problem worldwide. Aged cotton rats (*Sigmodon hispidus*) are considered novel model animals for studying CKD, especially as the females develop severe tubulointerstitial lesions with anemia. To investigate the renal pathologic features in aged male cotton rats and their characteristic glomerular injuries, the animals were divided into young, adult, old-aged, and advanced-aged groups (1–4, 5–8, 9–12, and 13–17 months, respectively) and pathologically analyzed. Anemia and renal dysfunction, as indicated by hematologic and serologic parameters, were significantly milder in the advanced-aged males than in the old-aged females. The males had increased urinary albumin-to-creatinine ratios from the old-age period, with the advanced-aged males having significantly higher levels than those in the old-aged females and young males. The old-aged females did not show clear glomerular injuries, whereas the advanced-aged males showed membranous lesions characterized by irregular and thickened glomerular basement membranes (GBMs). Characteristically, several large-sized projections from the GBM toward the podocytes were observed by microscopy, and podocytes covering these projections effaced their foot processes. The advanced-aged males showed aging-related IgG immune-complex depositions in the paramesangial regions and along the GBM. Furthermore, the positive reaction for podocin (a podocyte molecule) was granulated along the GBM. Thus, we clarified the albuminuria associated with altered glomerular structures in advanced-aged cotton rats, and that these phenotypes were closely associated with aging. These data help to clarify the aging-related pathogenesis of glomerular injury.

Introduction

Chronic kidney disease (CKD) is becoming a common and serious problem encountered in both human and veterinary medicine, given the growing trend toward the aging of society and the fact that the disease is associated with an older age in mammals (Bartges 2012; Coresh et al. 2007). The translational research based on Zoobiquity (Evans and Leighton 2014), targeting the elucidation of cross-species risk factors and pathogenesis, would be helpful in solving this common problem among mammalian species. In addition to genetic factors, several systemic factors also affect CKD development, such as infection, hypertension, and/or autoimmune disease-related conditions (Levey and Coresh 2012). Notably, in the Japanese people, glomerular diseases such as chronic glomerulonephritis, diabetic nephropathy, and renal sclerosis are the primary causes of CKD requiring dialysis (Masakane 2015), where men in particular tend to show a more rapid progression of the disease than women do (Silbiger and Neugarten 1995). Interestingly, in the veterinary area, the pathologic features of CKD differ among companion animals. For example, the glomerulus tends to be injured in dogs, whereas the tubulointerstitium is more often damaged in cats (Ichii et al. 2011).

The mammalian glomerulus has a crucial role in maintaining the glomerular filtration barrier. Structural disruption of the glomerulus causes proteinuria, a condition in which the urine is composed mainly of leaked serum albumin (hence called albuminuria). Among the cells that make up the glomerulus, the podocytes (i.e., glomerular epithelial cells) are important for the physiologic and pathologic regulation of the glomerular filtration barrier through their creation of a slit diaphragm, and for the maintenance of the glomerular basement membrane (GBM) together with the glomerular endothelial cells (Daehn 2018). The morphofunctional alteration of the glomerular filtration barrier critically affects the progression of CKD. In humans, the degree of proteinuria is closely and positively correlated with the increased incidence of end-stage renal failure (Iseki et al. 2003). Furthermore, the severity of proteinuria is negatively correlated with the survival scores both in dogs and cats (Rudinsky et al. 2018; Syme et al.

2006). To facilitate the future development of novel diagnostic and/or therapeutic strategies for CKD, genetically modified animals in which functional molecules of the podocyte or the induction of anti-glomerulus immunity are targeted have been mainly used to clarify the molecular pathogenesis of albuminuria (Yang et al. 2018), as spontaneous animal models of this disease are scarce.

The cotton rat (*Sigmodon hispidus*) is an experimental rodent originating from the southern United States. Many studies have reported that this rodent is associated with an increased susceptibility to pathogenic human viruses, protozoans, metazoans, bacteria (e.g., *Leishmania* and *Echinococcus*), and respiratory disease viruses (Blanco et al. 2014). In addition, unique disease phenotypes have been identified in cotton rats, including fragile tails, stomach cancer, and cardiomyopathy (Faith et al. 1997). In our previous studies, we had also identified some unique phenotypes of this species, such as the presence of pharyngeal pouch remnants, visceral adipose inflammation and ectopic fat accumulation in the pancreas without obesity, and the predominance of CKD in the females (Ichii et al. 2016, 2018; Nakamura et al. 2018, 2019). Furthermore, we clarified that CKD in aged female cotton rats was more severe with aging and strongly affected by female sex hormones and the development of pyometra (Ichii et al. 2018). The main renal histologic feature of female cotton rats is chronic tubulointerstitial nephritis characterized by the infiltration of immune cells and the dilation of distal tubules rather than glomerular lesions, whereas the males show only moderate tubulointerstitial lesions (Ichii et al. 2016, 2018). Furthermore, although glomerular lesions are also a major pathologic feature of CKD (Japanese Society for Dialysis Therapy 2015), their detailed features and change of the functions associated with the glomerulus, including membranoproliferative lesions, immune-complex deposition, and proteinuria, are still unclear.

In this study, we carefully examined the glomerular histopathology and identified the glomerular lesions characterized by GBM abnormality with albuminuria in male cotton rats of advanced age. Additionally, the renal dysfunction indicated by the increases in blood urea nitrogen (BUN) and serum

creatinine (sCr) levels was noted in aged females but not in males, although the males showed glomerular injury. Thus, this study suggested that cotton rats would be a suitable animal model for the study of spontaneous albuminuria with morphofunctional alteration of the GBM due to aging.

Materials and methods

Animals

The animal experimentation was performed according to the guidelines of the Hokkaido Institute of Public Health (Sapporo, Japan) (Approval No.: K27-03). Male and female cotton rats (1–21 months of age) were maintained as the HIS/Hiph strain through continuous inbreeding under conventional conditions at the Hokkaido Institute of Public Health. The animals were divided into four groups by age; namely, young (1–4 months of age), adult (5–8 months of age), old-aged (9–12 months of age), and advanced-aged (13–17 months of age). With the animals under deep isoflurane anesthesia, blood was collected from the vena cava, following which the animals were euthanized by severing of the abdominal aorta. The extracted serum was used for serologic analysis. The kidneys were fixed in 4% paraformaldehyde or in a 2% paraformaldehyde and 2.5% glutaraldehyde mixture for histopathologic analysis, and a part of the kidney was stored in Michel's Transport Medium (Polysciences, Warrington, PA, USA).

Blood examination

Hematologic analysis was performed using an XT-1800xi instrument (Sysmex, Kobe, Japan) for determination of the white blood cell (WBC) and red blood cell (RBCs) numbers, as well as the hemoglobin (Hgb) and hematocrit (HCT) values. For the serologic tests, the BUN and sCr levels were analyzed using a Fuji Dri-Chem 7000v analyzer (Fujifilm, Tokyo, Japan).

Urinalysis

Freshly voided urine was collected during 9 am and 5 pm on two separate days and pooled for urinalysis. The urinary albumin was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Approximately 10 μg of protein per sample was separated by SDS-PAGE at 225 V for 40 min on Novex 4–20% Tris-Glycine Mini Gels (Thermo Fisher Scientific, Waltham, MA, USA). Molecular weight markers ranging from 6.5 to 200 kDa (Bio-Rad, Hercules, CA, USA) and 0.5 μg of bovine serum albumin were included in each gel. The gels were stained with a Coomassie dye (GelCode Blue Stain Reagent, Thermo Fisher Scientific) and analyzed using image analysis software (CS Analyzer; ATTO, Tokyo, Japan). The urinary creatinine (uCr) level was measured by the alkaline picrate method (Creatinine Companion Kit; Exocell, Philadelphia, PA, USA) and used for the normalization of the urinary albumin level.

Histopathologic analysis

The paraffin-embedded kidney sections were stained with periodic acid Schiff (PAS) or periodic acid methenamine silver (PAM) for histopathologic analysis. Immunohistochemistry for podocin was performed to evaluate the slit diaphragm feature. In brief, the sections were deparaffinized, heated in Tris-HCl buffer (pH 9.0) for 15 min at 110℃, and incubated with anti-human podocin rabbit antibody (1:400; IBL, Gunma, Japan) followed by anti-rabbit IgG antibody (undiluted; Nichirei, Tokyo, Japan), according to a previously published streptavidin-biotin method (Ichii et al. 2016). The color was developed by incubating the sections in a 3,3′-diaminobenzidine tetrahydrochloride–hydrogen peroxide solution. The same concentration of normal rabbit IgG (Santa Cruz Biotechnology, Dallas, TX USA) was used as a primary antibody control to confirm whether there were any specific reactions from using this antibody.

158 For the immunofluorescence study, the kidney tissue preserved in Michel's Transport Medium was
159 washed with 0.01 M phosphate-buffered saline (PBS) and embedded into a Tissue–Tek O.C.T. compound
160 (Sakura Finetek, Tokyo Japan) with liquid nitrogen and isopentane. Frozen sections of 4-μm thickness
161 were prepared and air-dried completely. The sections were then incubated with either chicken anti-cotton
162 rat IgG antibody (1:3200; Immunology Consultants Laboratory, Portland, OR, USA) or rabbit anti-mouse
163 IgA antibody (1:3200; Bethyl Laboratories, Montgomery, AL, USA) at 4℃ overnight. A previous study
164 had suggested that there was cross-reactivity between mouse IgA and cotton rat IgA (Guichelaar et al.
165 2014). Then, the sections were washed with PBS and incubated with Alexa Fluor 488-conjugated donkey
166 anti-chicken IgY antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and
167 Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody (1:500; Thermo Fisher Scientific) at room
168 temperature for 30 min. After a wash with PBS, the sections were mounted and examined using a
169 BZ-X710 microscope (Keyence, Osaka, Japan).
170 For transmission electron microscopy, the kidneys were fixed in a 2% paraformaldehyde and 2.5%
171 glutaraldehyde mixture for 4 h, then post-fixed in 1% osmium tetroxide for 2 h, and finally embedded in
172 Quetol 812 resin (Nisshin EM, Tokyo, Japan). Ultrathin sections were then doubly stained with uranyl
173 acetate and lead citrate and observed using a JEM-1400 Plus microscope (JEOL, Tokyo, Japan).
174
175 *Statistical analyses*
176 The results are expressed as the median value. The Mann–Whitney U test was used to compare two
177 groups ($P < 0.05$). The Kruskal–Wallis test was used for comparing more than three populations, and
178 multiple comparisons were performed using Dunnett's test when a significant difference was observed (P
179 < 0.05). Spearman's correlation test ($P < 0.05$) was used to analyze the correlation between two
180 parameters.
181

182 **Results**

183 *Anemia and tubulointerstitial lesions are milder in males than in females*

184 We had already previously clarified that female cotton rats developed renal anemia (Ichii et al. 2016). In
185 this study, we also examined anemic parameters in the males and compared them with those of the
186 females (Fig. 1). As in our previous study, the RBC, HCT, and Hgb levels in the females had decreased
187 with aging (Fig. 1a-c). In the males, the RBC and Hgb levels were significantly more decreased in the
188 advanced-aged group than in the other groups (Fig. 1a and c), but the HCT levels were not significantly
189 changed among the groups (Fig. 1b). The RBC, HCT, and Hgb values tended to be higher in the males
190 than in the females, and significant sex-related differences were observed in the adult and old-aged
191 groups. When we compared these anemic parameters between the advanced-aged males and old-aged
192 females, the former showed significantly higher values for all parameters ($P < 0.001$). In contrast, no
193 clear age-related change was observed in the WBC counts of both sexes, but the old-aged female group
194 showed significantly higher values than those of the old-aged and advanced-aged male groups (Fig. 1d).

195 Our previous study had indicated that renal anemia in cotton rats seemed to be strongly affected by
196 the tubulointerstitial lesion (Ichii et al. 2016). As shown in Fig. 1e, the females developed severe
197 tubulointerstitial inflammation with dilation of the renal tubule at the corticomedullary junction, whereas
198 these lesions were clearly milder in the males, even in the advanced-aged group (Fig. 1e).

199

200 *Aged males developed albuminuria*

201 Fig. 2 shows the renal functional parameters. The BUN levels tended to increase with aging in both sexes,
202 and the females showed significantly higher values than those of the males in the old-aged groups (Fig.
203 2a). Furthermore, the advanced-aged males showed significantly higher values relative to those of the
204 young and old-aged groups. The sCr levels tended to be increased with aging in the females rather than in

the males, with the old-aged and advanced-aged female groups showing significantly higher values (Fig. 2b).

Next, we used SDS-PAGE to examine the urinary albumin excretion levels in the males (Fig. 2c). As shown in the figure panels, the clear band detected at approximately 66 kDa indicated that the albumin (and its band intensity) level tended to be higher in the aged males. With regard to the quantified urinary albumin-to-creatinine ratio (uACR), the values increased with aging in the males, where the advanced-aged males showed significantly higher values than those of the young males ($P < 0.001$, Fig. 2d). Furthermore, the advanced-aged males also showed significantly higher values than those of the old-aged females. As shown in Fig. 2e, the uACR was significantly and positively correlated with age in the males ($\rho = 0.804$, $P < 0.001$).

Aged males developed glomerular membranous lesions

Fig. 3 shows the glomerular histopathologic features in the cotton rats. Males of young age had a normal glomerulus structure, where no proliferative or membranous lesions were observed (Fig. 3a). Furthermore, as reported previously (Ichii et al. 2016), the females in all the age groups examined also showed no clear glomerular lesions, but cell infiltrations with fibrous lesions were observed around the renal corpuscles (Fig. 3b). In contrast, the aged males manifested glomerular lesions that were characterized by thickened or wrinkled GBMs in the PAS-stained sections, and these lesions were clearer in the advanced-aged group (Fig. 3c and d). Compared with the GBM alterations, proliferative lesions were scarce or mild in the advanced-aged males. Interestingly, in this advanced-aged group of males, the high-magnification image showed PAS-positive rod-like structures along the GBM that were projected toward the podocytes (Fig. 3e). These rod-like structures were clearly observed in black color in the PAM-stained section indicating the components of the GBM (Fig. 3f) and were larger than the spikes observed in membranous nephropathy.

Males showed age-related immune-complex deposition and podocyte alteration

As shown in Fig. 4a, the young male cotton rats did not show any positive reactions for IgG or IgA in the immunofluorescence assay using snap-frozen sections. In contrast, the advanced-aged males showed positive reactions for IgG, but not for IgA, which were observed mainly in the paramesangial regions and along the GBM (Fig. 4b). Fig. 4c shows the localization of podocin, a slit diaphragm molecule, where a positive reaction was clearly observed along the GBM in the young group of males. In contrast, these reactions appeared as granulated patterns of various sizes in the advanced-aged males.

Males showed age-related alteration of the GBM

Fig. 5 shows the ultrastructure of the glomerulus in the advanced-aged group of male cotton rats. Similar to the light microscopy observations, projections from the GBM toward the podocytes were clearly observed (Fig. 5a and b). Furthermore, the thickness of the GBM differed among the glomerular capillary rete, being either thin (Fig. 5c) or thickened with large-sized projections (Fig. 5d). In the latter case, the foot processes of the podocytes covering the GBM were effaced. In the other lesions, the incorporation of the mesangial matrix into the paramesangial regions (Fig. 5e) and the reticular pattern of the GBM (Fig. 5f) were also observed.

Discussion

In this study, we found that advanced-aged male cotton rats developed albuminuria without severe renal dysfunction, as indicated by the remarkable elevations of BUN or sCr. As previously shown in aged rodents, including mice and rats, albuminuria was generally observed with glomerular injury and reduction of the glomerular filtration rate (Alt et al. 1980; Lim et al. 2012). As a common feature between aged humans and animals, the kidneys show membranous lesions characterized by GBM thickening and wrinkling associated with the loss of the capillary loops, as well as proliferative lesions, such as mesangial matrix expansion due to the imbalanced production of extracellular matrixes, and these age-related glomerular changes are more apparent in males than in females (Anderson and Brenner, 1986; Zhou et al. 2008). Although cotton rats seemed to develop the same age-related kidney damage as found in other rodent species, clear sex-related differences were characteristically observed in their phenotypes. In brief, whereas aged female cotton rats developed severe renal inflammation with renal anemia and renal dysfunction (Ichii et al. 2016, 2018), these phenotypes were milder in male cotton rats. Furthermore, we had also already clarified that pyometra, female sex hormone changes, and induction of the estrogen receptor in the renal tubules regulated the tubulointerstitial lesions in female cotton rats (Ichii et al. 2018). These CKD-associated phenotypes found in the females were inhibited by ovariectomy, indicating the exacerbative activity of the female sex hormones toward the tubulointerstitial lesions (Ichii et al. 2018). In addition, we examined the effects of male sex hormones on the development of albuminuria in advanced-aged males by castrating the cotton rats at 2 months before sampling (at ~10.5 months of age, n = 4). As a result, because there was no change in the albuminuria by castration (Supplemental Fig. S1), we surmised that the male sex hormones contributed poorly to the phenotype observed in the advanced-aged males. Therefore, the glomerular damage in the male cotton rats was strongly affected by aging, whereas the renal pathogenesis in the female cotton rats was more strongly affected by the female sex hormones or reproductive organ abnormalities, as we had reported previously.

In general, morphofunctional changes of the glomerular filtration barrier cause leakage of the serum protein into primarily the urine, resulting in albuminuria (Anderson and Brenner. 1986; Yumura et al. 1989). From the observed histopathologic features of the glomerular filtration barrier in advanced-aged male cotton rats, we clarified the presence of 1) GBM alterations, 2) the deposition of IgG-related immune complexes, and 3) podocyte injuries. As commonly found among aged rodents (Yumura et al. 1989), the deposition of immune complexes (particularly IgG) into the glomerulus tends to increase with aging. Furthermore, the GBM of the advanced-aged males showed no immune-mediated lesions, such as spike-like structures and double contouring, or mesangial proliferative lesions like those found in membranous nephropathy and proliferative glomerulonephritis. Therefore, the IgG depositions found in the glomerulus of the advanced-aged males would not be immune-mediated glomerular lesions but aging-related ones, as commonly found in other rodent species. Therefore, we concluded that the direct causal factors of albuminuria in the advanced-aged male cotton rats were the changes in podocyte morphology characterized by foot process effacement and altered expression of the slit diaphragm protein podocin, as well as GBM alterations.

In particular, the morphologic alterations of the GBM, characterized by large projections toward the podocytes and random thickness, were characteristic features in the glomerulus of the advanced-aged male cotton rats. Importantly, in these lesions, effacement of the podocyte foot processes was clearly observed. Interestingly, the large projections of the GBM toward the podocytes were clearly different from the “spike lesions” found in the immune-complex depositions in the GBM. However, there are no clear reports related to these large-sized GBM projections in humans or in other laboratory rodents. The GBM is coordinately produced and maintained by the podocytes and glomerular endothelial cells, and is mainly composed of laminin, type IV collagen, nidogen, and heparan sulfate proteoglycan (Miner 2012). In particular, Abrahamson et al. (2009) indicated the importance of the podocytes for the production of collagen α3α4α5 (IV). Therefore, there is a possibility that cotton rats might have a genetic deficiency

(e.g., Alport syndrome) associated with the production and regulation of the GBM components. However, we surmise that these genetic variants or mutations would be less likely to contribute to the development of GBM abnormality in this species, because cotton rats do not develop GBM abnormality from a young age, as found in genetic mutation diseases, whereas the advanced-aged individuals do, especially the males. In fact, there are no data on the development of hearing loss and eye abnormalities in these rats, as found in patients with Alport syndrome. Furthermore, altered systemic conditions such as high-pressure and/or diabetic conditions also affect the morphologic alteration of the GBM. In fact, we had already reported that aged cotton rats develop metabolic disorders associated with visceral adipose inflammation and fatty pancreas without obesity, characterized by hyperinsulinemia, hyperglycemia, and dyslipidemia (Nakamura et al. 2019). Therefore, the histopathologic features of the GBM abnormality in the advanced-aged male cotton rats would be formed by several complex factors, including aging, genetic factors, and/or altered systemic conditions.

In conclusion, we found the development of albuminuria with glomerular abnormalities characterized by GBM morphologic alterations (viz., random thickness and large projections toward the injured podocytes) in the advanced-aged male cotton rats. The clarification of the pathogenesis of spontaneous albuminuria in this rodent species would contribute to our understanding of the development of age-related injuries of the glomerular filtration barriers in humans and other animals.

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Compliance with ethical standards

Conflict of interest All the authors of this paper have no conflicts of interest to declare.

Statement of human and animal rights The animal experimentation was performed according to the guidelines of the Hokkaido Institute of Public Health, Sapporo, Japan (Approval No.: K27-03).

Author contributions

O.I., T.N., T.I., T.H., Y.H.A.E., and Y.K. conceived and designed the experiments. O.I., T.N., T.I., Y.O., M.H., M.A.M., and R.M.I. performed the experiments. O.I., T.N., and Y.S. analyzed the data. O.I. and Y.K. wrote the paper.

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391

Figure legends

Fig. 1 Hematologic parameters of cotton rats

a. Red blood cells (RBC).

b. Hematocrit (HCT).

c. Hemoglobin (Hgb).

d. White blood cells (WBC).

M: male; F: female; A.aged: advanced-aged group. *, **, ***: significance with A.aged group (P < 0.05, 0.01, 0.001). ##, ###: significance with males in the same groups (P < 0.01, 0.001). †††: significance with old-aged females (P < 0.001).

e. Histopathologic features of the corticomedullary junction in cotton rats.

The old-aged females showed severe tubulointerstitial lesions, such as dilated tubules (asterisks) and cell infiltrations (arrows). In contrast, these lesions were mild in the A.aged males. PAS staining. Bars = 500 μm.

Fig. 2 Renal function parameters of cotton rats

a. Blood urea nitrogen (BUN).

b. Serum creatinine (sCr).

M: male; F: female. A.aged: advanced-aged group. *: significance with A.aged group (P < 0.05). ###: significance with males in the same groups (P < 0.001). †††: significance with old-aged females (P < 0.001).

c. Urinary albumin. Urinary protein in the males was analyzed by SDS-PAGE. The strong bands detected are approximately 66 kDa in size, similar to that of bovine serum albumin (BSA). m:

months. The band intensities for the old-aged (9 m) or advanced-aged males (14–16 m) were stronger than those of young or adult males (3–5 m).

d. Urinary albumin-to-creatinine ratio (uACR).

M: male; F: female. A.aged: advanced-aged group. ***: significance with A.aged group ($P < 0.001$). †: significance with old-aged females ($P < 0.05$).

e. Correlation between uACR and age.

The male groups were analyzed (n = 20). ρ: Spearman's correlation coefficient ($P < 0.001$).

Fig. 3 Glomerular pathology of cotton rats

a. Young male. The glomerulus shows normal structures. Bar = 30 μm.

b. Advanced-aged (A.aged) female. The glomerulus structures are apparently normal, and cell infiltration and fibrosis are observed around the renal corpuscle (asterisks). Bar = 30 μm.

c and d. A.aged male. Membranous lesions characterized by a thickened glomerular basement membrane (GBM) are noted. Bars = 30 μm.

e and f. A.aged male. Along with the GBM, PAS- or PAM-positive large granules or projections toward the podocytes are evident. Bars = 15 μm.

PAS staining: a–e. PAM staining: f.

Fig. 4 Immune-complex deposition and podocyte molecules in the glomerulus of cotton rats

a. Immunofluorescence assay of IgG and IgA in young male cotton rats. No immune-positive reactions were observed for IgG or IgA. Blue: nuclear Hoechst staining. Bars = 50 μm.

b. Immunofluorescence assay of IgG and IgA in advanced-aged (A.aged) male cotton rats. Immune-positive reactions are observed for IgG, but not for IgA. IgG-positive reactions are observed

along with the glomerular basement membrane and paramesangial regions. The square indicates the magnified area. Green: IgG- or IgA-positive reaction. Blue: nuclear Hoechst staining. Bars = 50 μm.

c. Immunohistochemistry for podocin. The young male shows clear and linear podocin-positive reactions. The A.aged male shows granular patterns. The square indicates the magnified area. Bars = 50 μm.

Fig. 5 Ultrastructures of the glomerulus in advanced-aged (A.aged) cotton rats

a and b. Large-sized projections of the glomerular basement membrane (GBM). Single or several projections of the GBM toward the podocytes are observed (arrows). Bars = 30 μm.

c and d. Irregular thickness of the GBM. Thinned or thickened GBMs are observed. In the latter case, large projections are also noted (arrows). Bars = 30 μm.

e and f. Other characteristics of the glomerulus. Mesangial matrix incorporation at the paramesangial regions (arrowheads) and reticular patterns of the GBM (asterisks) are observed.

Pod: podocytes. Cap: capillary. Bars = 30 μm.

Supplemental Fig. S1 Castration analysis of male cotton rats

a. Time course of castration (Cast) and sampling.

b. Urinary protein-to-creatinine ratio (uPCR) and urinary albumin-to-creatinine ratio (uACR). N.S.: not significant

Fig.1

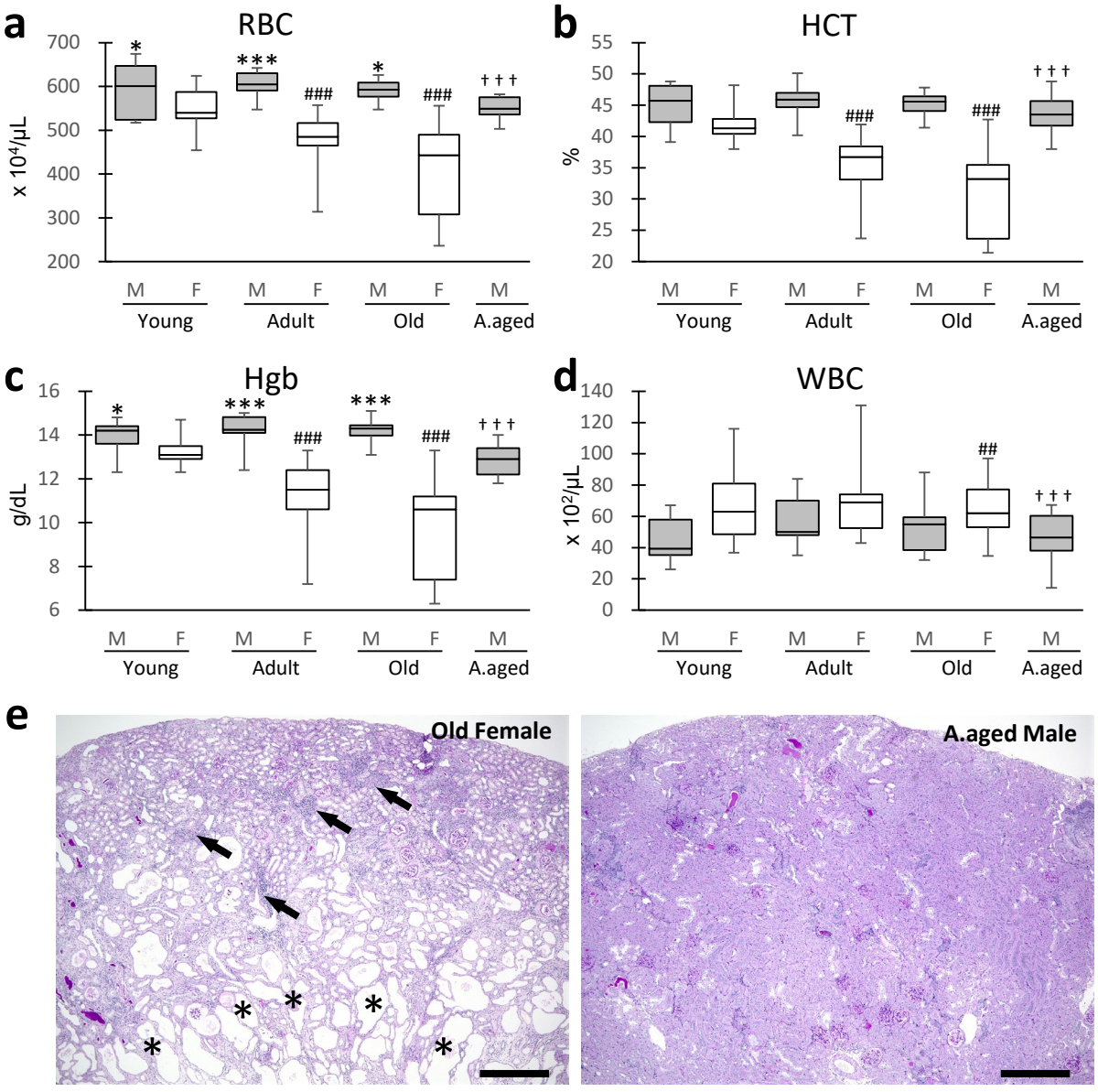

a
RBC
x 10⁴/μL
700
600
500
400
300
200
b
HCT
%
55
50
45
40
35
30
25
20
c
Hgb
g/dL
16
14
12
10
8
6
d
WBC
x 10²/μL
140
120
100
80
60
40
20
0
M F M F M F M
Young Adult Old A.aged
e
Old Female
A.aged Male

Fig.2

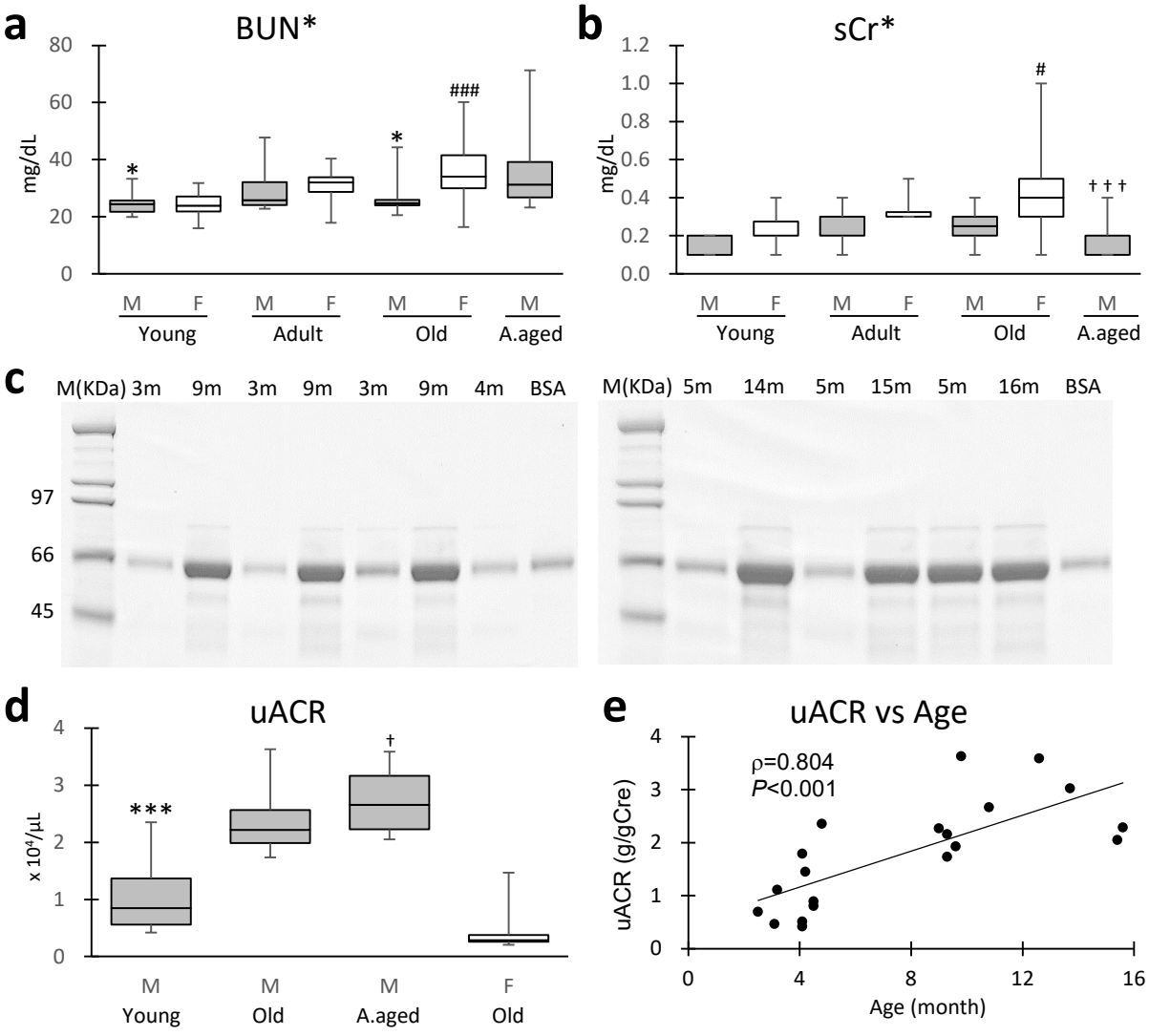

a
BUN*
mg/dL
80
60
40
20
0
*
*
###
M F M F M F M
Young Adult Old A.aged
b
sCr*
mg/dL
1.2
1.0
0.8
0.6
0.4
0.2
0.0
#
† † †
M F M F M F M
Young Adult Old A.aged
c
M(KDa) 3m 9m 3m 9m 3m 9m 4m BSA
M(KDa) 5m 14m 5m 15m 5m 16m BSA
97
66
45
d
uACR
x 10⁴/μL
4
3
2
1
0
***
†
M Young
M Old
M A.aged
F Old
e
uACR vs Age
ρ=0.804
P<0.001
uACR (g/gCre)
4
3
2
1
0
0 4 8 12 16
Age (month)

Fig.3

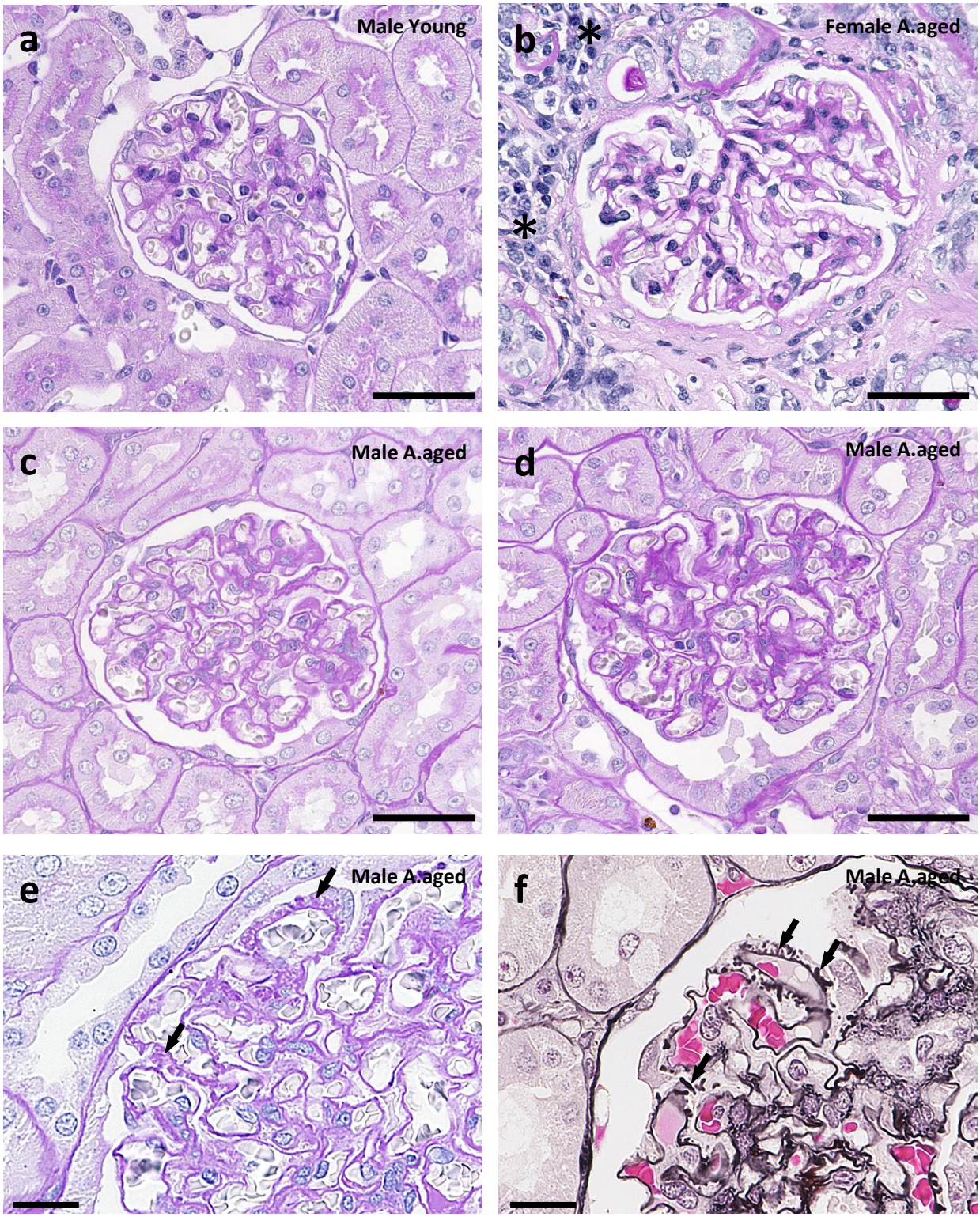

a
Male Young
b
*
*
Female A.aged
c
Male A.aged
d
Male A.aged
e
Male A.aged
f
Male A.aged

Fig.4

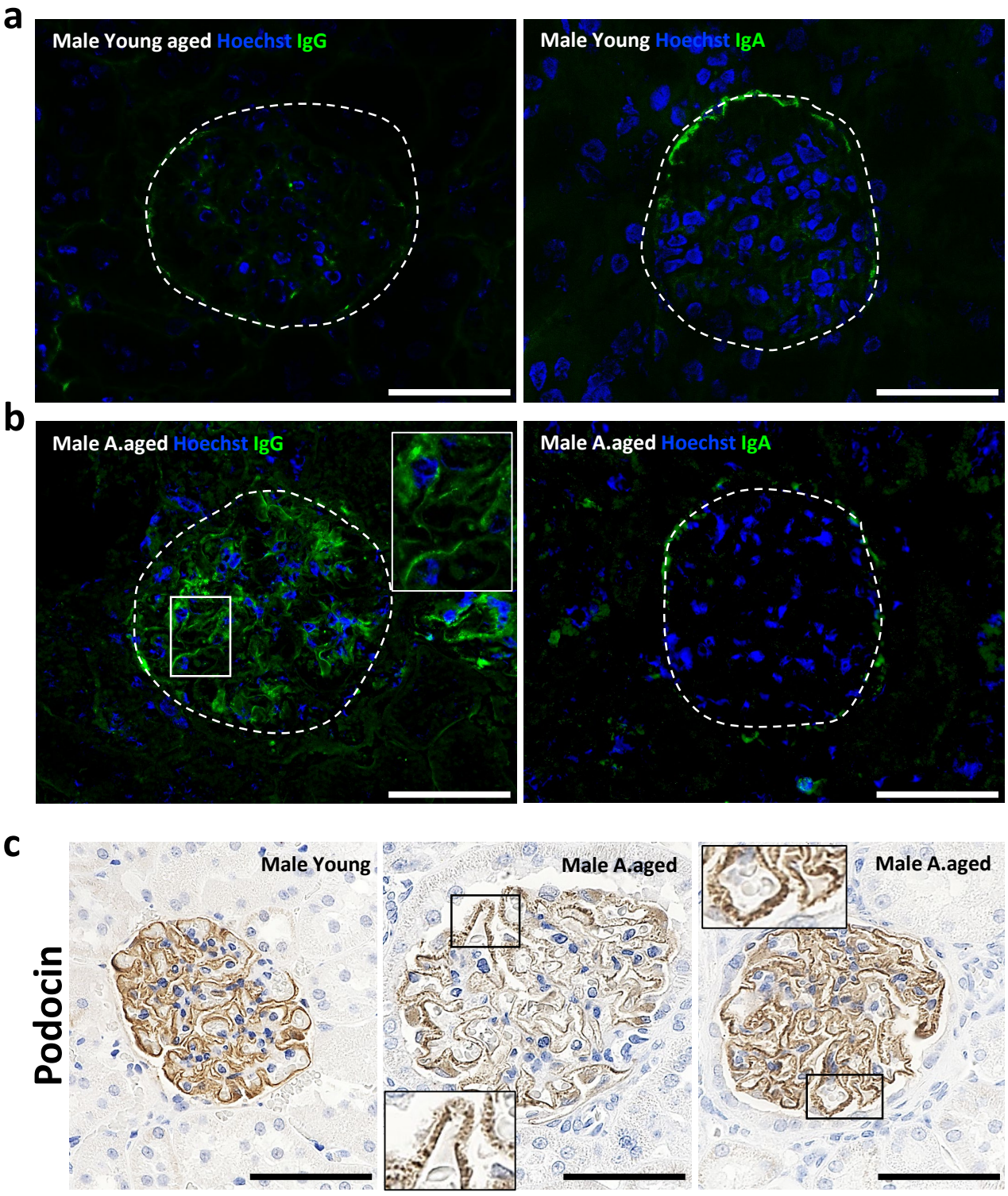
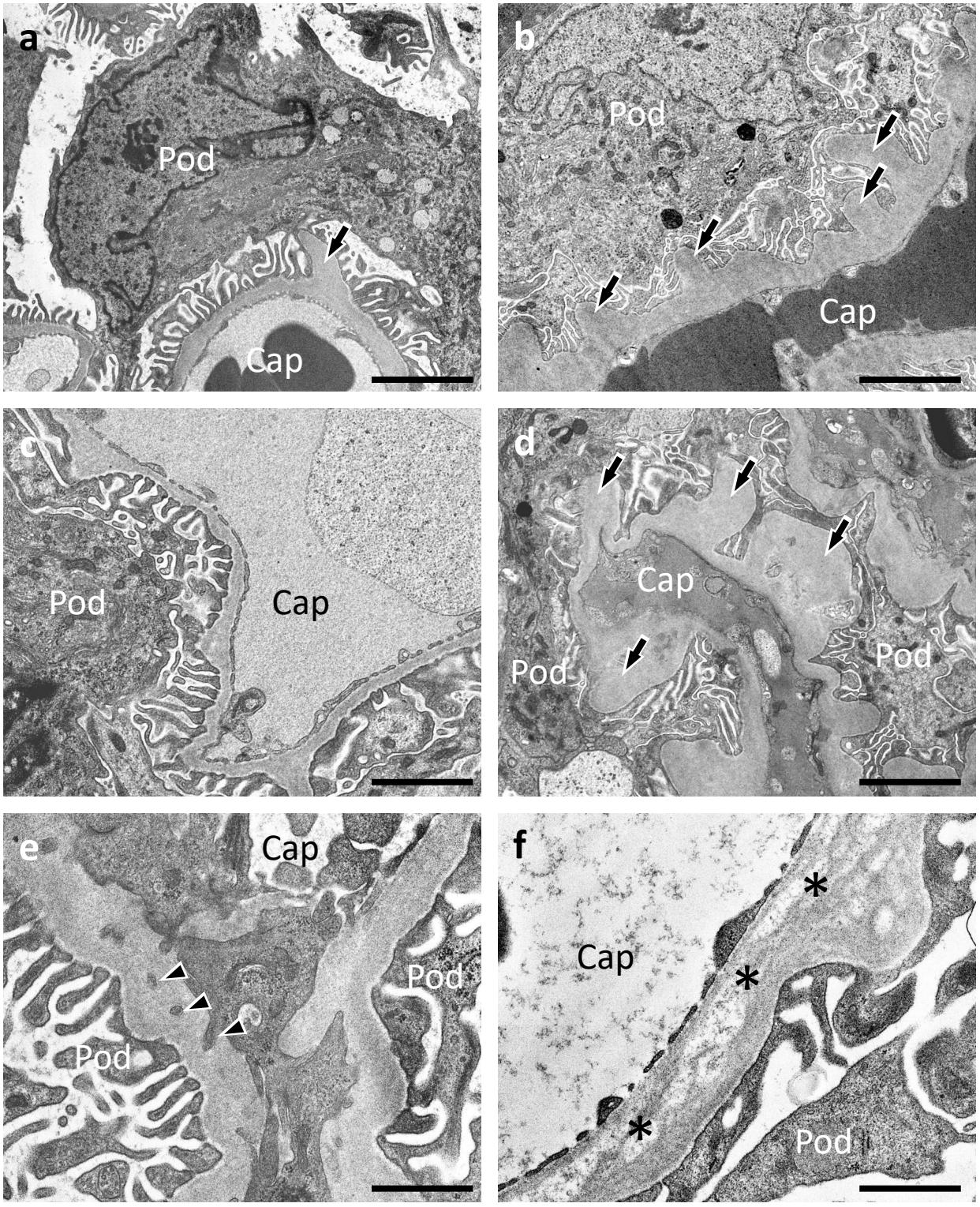

a
Male Young aged Hoechst IgG
Male Young Hoechst IgA
b
Male A.aged Hoechst IgG
Male A.aged Hoechst IgA
c
Podocin
Male Young
Male A.aged
Male A.aged

Fig.5



Supplemental Fig. 1

