Sex differences in renal inflammation and injury in high fat diet fed Dahl salt sensitive rats.

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Running title: Sex in hypertension and renal injury/inflammation

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Key Words:

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Supplemental Methods:

Measurement of mean arterial blood pressure MAP:

Procedures used for implantation of BP telemeters in rats have been described previously[1]. After 2-4 weeks on the CD or HFD radiotelemeters (HD-S210, Data Sciences International, St. Paul, MN) were implanted under isoflurane anesthesia (2-3%). After the rats recovered from surgery ($\geq$ 3 days), blood pressure was sampled continuously for 10 seconds every 10 minutes. 24 hours of arterial blood pressure (MAP) data were collected once weekly for up to 24 weeks.

Contributions of the sympathetic nervous system to control of BP.

At 24 weeks of feeding, neurogenic pressor activity was evaluated by measuring the maximal changes in MAP within 30 minutes after injection of hexamethonium (HEX, a nicotinic ACh receptor antagonist, 30 mg/kg, ip).

Histological assessment of renal injury:

Morphological assessments of renal injury and arterial structure were performed in paraffin-embedded slices by Masson's trichrome staining. Stained whole kidney sections were scanned and digitized with a slide scanner (VS110, Olympus America, Center Valley, PA).[2] Renal injury was semi-quantified blindly by a veterinary pathologist following a guideline of international standardized nomenclature for classifying lesions observed in rodent urinary systemic pathology.[3] Renal injury was identified and characterized as: hyaline cast (tubular proteinosis); interstitial fibrosis (peritubular fibrosis); glomerular sclerosis, and tubular atrophy. Injury severity scores were determined as: 0) no lesions; 1) <10% (minimal); 2) ≥10-25% (mild); 3) ≥25-50% (moderate); 4) ≥50% (severe). Scores of renal arterial hypertrophy and perivascular fibrosis were also semi-quantified blindly.

Histological assessment of renal inflammatory responses:

To assess and differentiate immune cell infiltration, paraffin-embedded kidney slices were stained for detecting monocytes/macrophages (rabbit anti-CD68, 1:100, MAB1435, Millipore) and T-cells (rabbit anti-CD3, 1:500, Ab5690, Abcam, Cambridge, MA). To assess the role of T-reg cell infiltration, tissue slices were co-immuno-stained with T-cell (rabbit anti-CD3, 1:500, Ab5690, Abcam) and FoxP3 (rabbit anti-FoxP3, 1:250, NB100-39002, Novus Biologicals, Littleton, CO). To verify the specificity of the primary antibody, immune-staining without primary antibodies was used as negative control, and the staining in slices from rat spleen was used as a positive control.

Immuno-stained whole renal sections were scanned and digitized with a slide scanner as described above. For quantification of $CD68^+$, $CD3^+$ and $CD3^+Foxp3^+$ cell infiltration into the cortex and medulla, digitized images of each section were selected as regions of interest and 20% of each section was captured at 200X magnification by systematic random sampling using stereological methods with newCAST software (version 5.2.1.1485; VisioPharm, Hoersholm, Denmark),[2] ~100 images were captured from each section. Captured images (0.8 x 0.8 μm/each region) were visualized using Stepanizer software (www.stepanizer.com), $CD68^+$, $CD3^+$ and $CD3^+Foxp3^+$ positive cells in each region were counted blindly. Total positive cell counts from all captured region were averaged by image numbers. Cell counting was also distinguished in cortex and medulla from captured images.

Blood sampling and tissue collection:

After 10, 17 or 24 weeks of CD or HFD, rats were anesthetized (2.5% to 3% isoflurane) after an overnight fast. Blood glucose was measured in triplicate in tail vein blood samples (Glucose Meter, FreeStyle Insulinx, Abbott, USA). Blood was also collected through the left ventricle, and centrifuged to separate the plasma, which was frozen at -80 °C for later analyses. Body weight (BW), and the weight of gonadal fat, retroperitoneal fat, kidney and left ventricle were recorded. The heart was extracted and the pulmonary arteries,

aorta and atria were carefully removed. Then the heart was weighed as whole heart, and left ventricle weight was measured after removal of the right ventricle. Kidneys were fixed in 4% paraformaldehyde for 48 hours and stored in 75% ethanol prior to embedding in paraffin blocks and histological and immunohistological staining. Cortex and medulla were also separated from kidneys, cut into small pieces and preserved in Allprotect Tissue Regent (Qiagen Inc, 76405, Germany) at 4°C for immediate stabilization of RNA in tissues; these preserved tissues were used subsequently for qtRT-PCR analysis.

Metabolic and biochemistry measures:

Plasma levels of cholesterol, triglycerides, blood urea nitrogen (BUN), creatinine and albumin were measured using a veterinary chemistry analyzer (Catalyst One Chemistry Analyzer, IDEXX Laboratories, Maine, USA). Plasma insulin and leptin were measured using a rat insulin ELISA kit (Mercodia, 10-1250-01, UPPSALA, Sweden) and rat leptin ELISA kit (Abcam, ab100773, MA, USA).

Detecting renal cytokines and MCP-1 expressions by qRT-PCR:

To determine the expression of renal cytokines and MCP-1, preserved renal cortex and spleen samples (as positive control) were homogenized on a bullet blender (Model: BBX24, Next Advance) with 0.9 - 2 mm stainless steel blades (SSB14B, Next Advance). Total RNA was extracted with RNA Mini Kit (#74104, Qiagen) and cDNA synthesis was done with High Capacity RNA to cDNA kit (4387406, ThermoFisher Scientific) per manufacturer's instructions. Real-time PCR reactions were set up as follows: (1) 10 μl of Fast Taqman Advanced MasterMix (4444558, ThermoFisher), (2) 1 μl of Taqman primer (4331182, ThermoFisher Scientific) (IL-6, Rn01410330_m1; IL-10, Rn01483988_g1, IL-17, Rn01757168_m1; TNF-α, Rn01525859_g1; MCP-1, Rn00580555_m1 and GAPDH, Rn01775763_g1), (3) 7 μl of molecular grade H_2O, (4) 2 μl of sample. RT-PCR reactions were run on the ABI 7500 (Applied Biosystems). GADPH was used as an internal control. The threshold was set to 0.1 for determining Ct value. Delta Ct values were calculated with the following formula and then averaged in each group: $\Delta Ct = Ct_{target} - Ct_{Gapdh.}$

References:

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Table S1: Biological measures from male and female Dahl SS rats fed CD and HFD with normal salt at 24 weeks of feeding.

Biological measures	CD-M (n=10)	HFD-M (n=9)	CD-F (n=5)	HFD-F (n=4)
Body weight (g)	457 ± 9	485 ± 8	298 ± 8	318 ± 5
Kidney/BW (%)	0.33 ± 0.007	0.35 ± 0.009	0.30 ± 0.008	0.31 ± 0.003
LV/BW (%)	0.25 ± 0.005	0.27 ± 0.006*	0.25 ± 0.009	0.28±0.004*
LV/LV+RV (%)	0.81 ± 0.004	0.84 ± 0.006*†	0.81 ± 0.01	0.81 ±0.01
Liver/BW (%)	2.6 ± 0.07	2.7 ± 0.1	2.1 ± 0.08	2.4 ± 0.08*
Gonadal fat (g)	4.9 ± 0.4	6.8 ± 0.6*	4.1 ± 0.3	6.3 ±1.2*
Retroperitoneal fat (g)	5.2 ± 0.4	8.3 ± 0.3*	4.9 ± 0.5	5.3 ± 1.2
Glucose (mg/dL)	91 ± 5	90 ± 4	98 ±12	93±8
Leptin (ng/ml)	0.51 ± 0.06	0.81 ± 0.1*	0.42 ± 0.04	0.65±0.08*
Insulin (ng/ml)	1.4 ± 0.6	1.9 ± 0.5	0.87 ± 0.4	0.86 ± 0.2
Cholesterol (mg/dl)	54 ± 3	74 ± 7	67±17	87±17
Triglycerides (mg/dl)	126 ± 8	106 ± 8	79 ± 16	82 ± 19
BUN (mg/dl)	15 ± 1	12 ± 1	18 ± 1	21 ± 5
Creatinine (mg/dl)	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
BUN/ Creatinine	55 ± 2	39 ± 2	52 ± 3	41 ± 4
Albumin (g/dl)-24Wks	2.6 ± 0.0	2.4 ± 0.0*†	2.7 ± 0.1	2.6 ± 0.1

BW, body weight; LV, left ventricle; RV, right ventricle; Alb, albumin; * $P<0.05$, CD vs HFD; † $P<0.05$ M vs F, unpaired *t*-test

Table S2: Blood pressure (Systolic, diastolic, mean blood pressure) and heart rate measures from male and female Dahl SS rats fed CD and HFD with normal salt at 10, 17 and 24 weeks of feeding.

BP and HR	CD-M (n=10)	HFD-M (n=9)	CD-F (n=5)	HFD-F (n=4)
10-WKs				
SBP (mmHg)	143 ±1	150 ± 1*	141 ± 4	149 ± 3
DBP (mmHg)	100 ± 1	104 ± 1	94 ± 3	99 ± 2
MAP (mmHg)	119 ± 1	125 ± 2	115 ± 3	121 ± 2
HR (bpm/min)	386 ± 3	390 ± 3	394 ± 2	384 ± 1
17-WKs				
SBP (mmHg)	149 ± 2	168 ± 4*	150 ± 7	168 ± 5*
DBP (mmHg)	103 ± 1	117 ± 4*	100 ± 5	115 ± 3*
MAP (mmHg)	123 ± 2	140 ± 4*	122 ± 6	138 ±4*
HR (bpm/min)	362 ± 3	366 ± 2	370 ± 7	358 ±2
24-WKs				
SBP (mmHg)	157 ± 6‡	193 ± 4*‡	154 ± 8‡	189 ± 6*‡
DBP (mmHg)	100 ± 5	137 ± 4*‡	103 ± 6	133 ± 3*‡
MAP (mmHg)	131 ± 6§	158 ± 4*‡	125 ± 7‡	156 ± 5*‡
HR (bpm/min)	362 ± 4	373 ± 3	364 ± 4	363 ± 1

SBP, systolic blood pressure; DBP, diastolic blood pressure, MAP, mean arterial blood pressure, WKs, weeks of diet feeding. * $P<0.05$, CD vs HFD; ‡ $P<0.05$, 24Wks vs 10 WKs; unpaired *t*-test.

Table S3: Biological measures from male and female SD rats fed CD and HFD with normal salt at 24 weeks of feeding.

Biological measures	CD-M (n=4-5)	HFD-M (n=4-5)	CD-F (n=4)	HFD-F (n=4)
Body weight (g)	676 ± 35	784 ± 49*	306 ± 18	341 ± 21
Kidney/BW (%)	0.25 ± 0.04	0.24 ± 0.01	N/A	N/A
LV/BW (%)	0.18 ± 0.004	0.16 ± 0.001	0.21 ± 0.004	0.19 ± 0.005
LV/LV+RV (%)	0.81 ± 0.008	0.80 ± 0.004	NA	NA
Gonadal fat (g)	12.6 ± 1.4	18.3 ± 0.9*	N/A	N/A
Retroperitoneal fat (g)	24.6 ± 4.2	47.9 ± 9.7*	N/A	N/A
Glucose (mg/dL)	75 ± 3	90 ± 4	149 ±12	116±8
Leptin (ng/ml)	1.4 ± 1.1†	4.2 ± 0.9*†	0.37 ± 0.1	1.2 ± 0.2*
Insulin (ng/ml)	0.82 ± 0.24†	0.84 ± 0.16†	0.20 ± 0.05	0.16 ± 0.01

BW, body weight; LV, left ventricle; RV, right ventricle; N/A, data are not available. * $P<0.05$, CD vs HFD; † $P<0.05$ M vs F, unpaired *t*-test

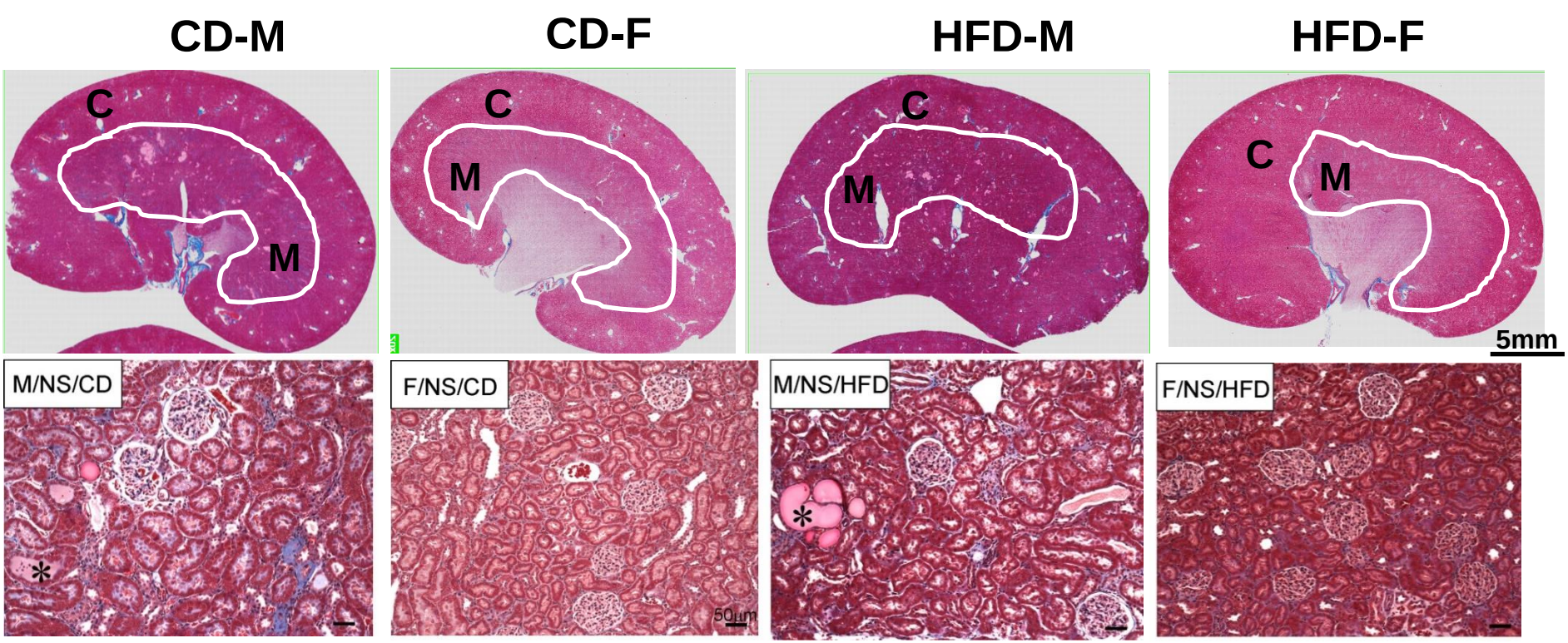


Fig S1: Representative light photomicrographs taken from Masson's Trichrome-stained whole renal sections and higher magnified cortical areas from male and female Dahl SS rats at 10 weeks (10WKs) of feeding. *indicate tubular proteinosis in kidney. C, cortex; M, medulla.

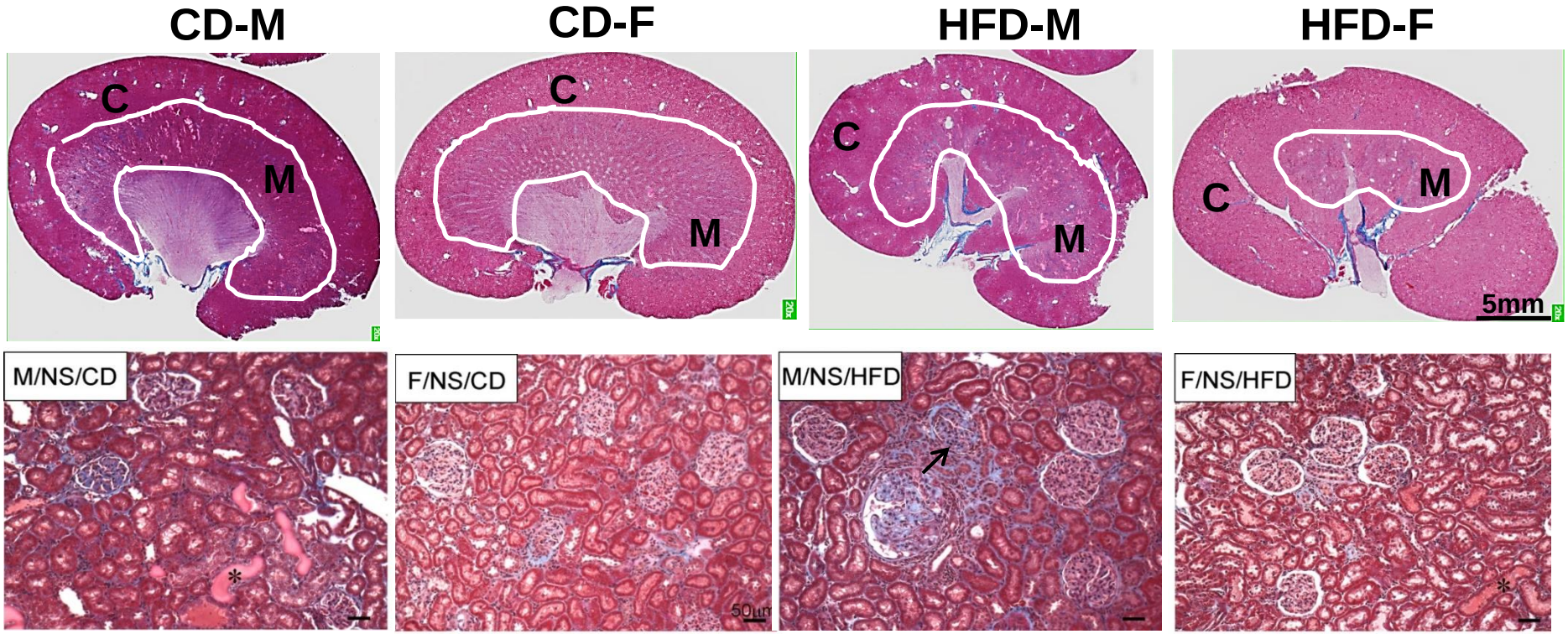


Fig S2: Representative light photomicrographs taken from Masson's Trichrome-stained whole renal sections and higher magnified cortical areas from male and female Dahl SS rats at 17 weeks (17WKs) of feeding. Indicating the occurrence of histological changes in kidney section: *tubular proteinosis; ←interstitial fibrosis. C, cortex; M, medulla.

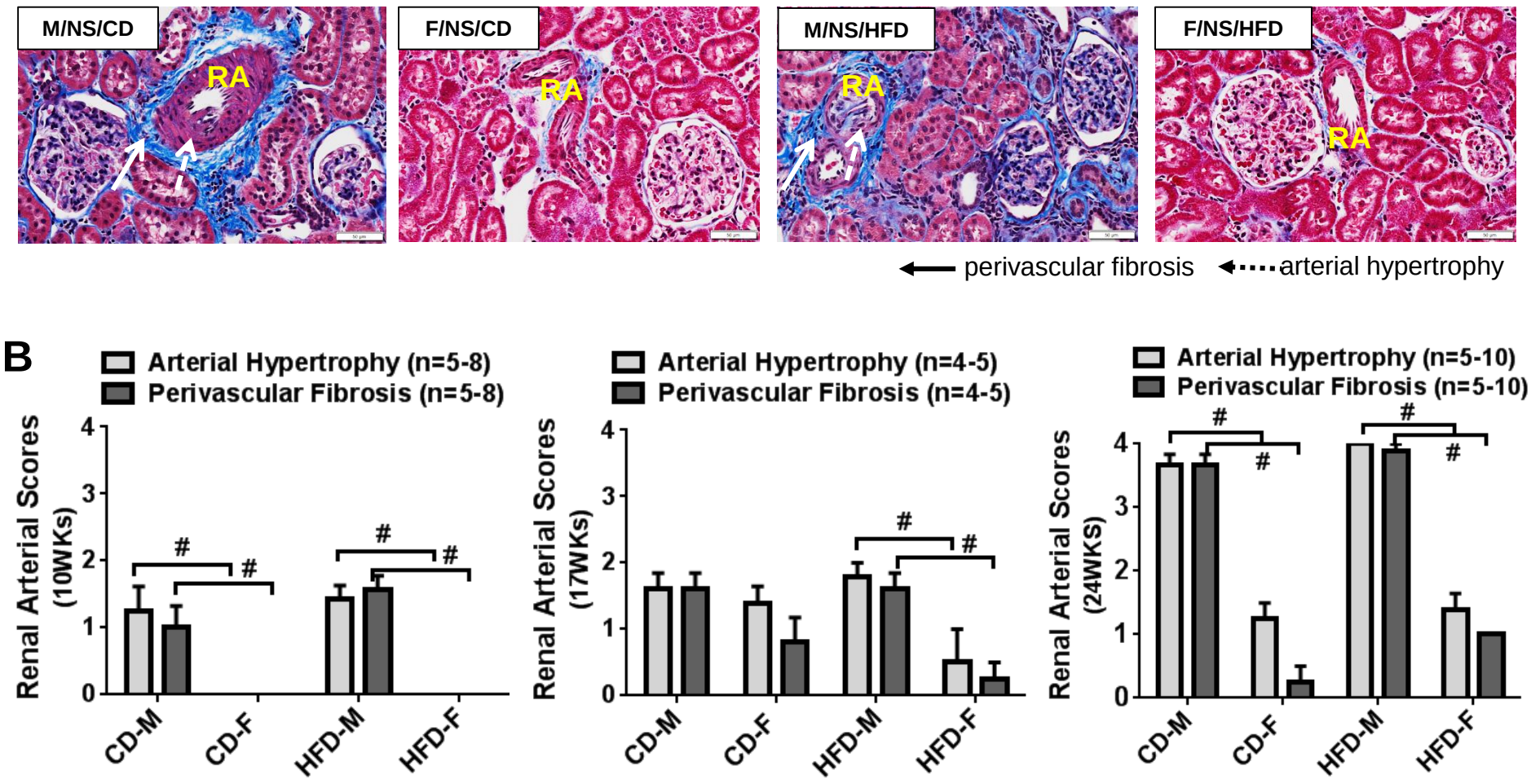


Fig S3: A. Representative light photomicrographs taken from Masson's Trichrome-stained higher magnified cortical areas from male and female Dahl SS rats at 24 weeks of feeding. RA, renal artery. B. Renal arterial hypertrophy and fibrosis scores in CD and HFD males and females at 10, 17 and 24 weeks of feeding. # P<0.05, M vs F.

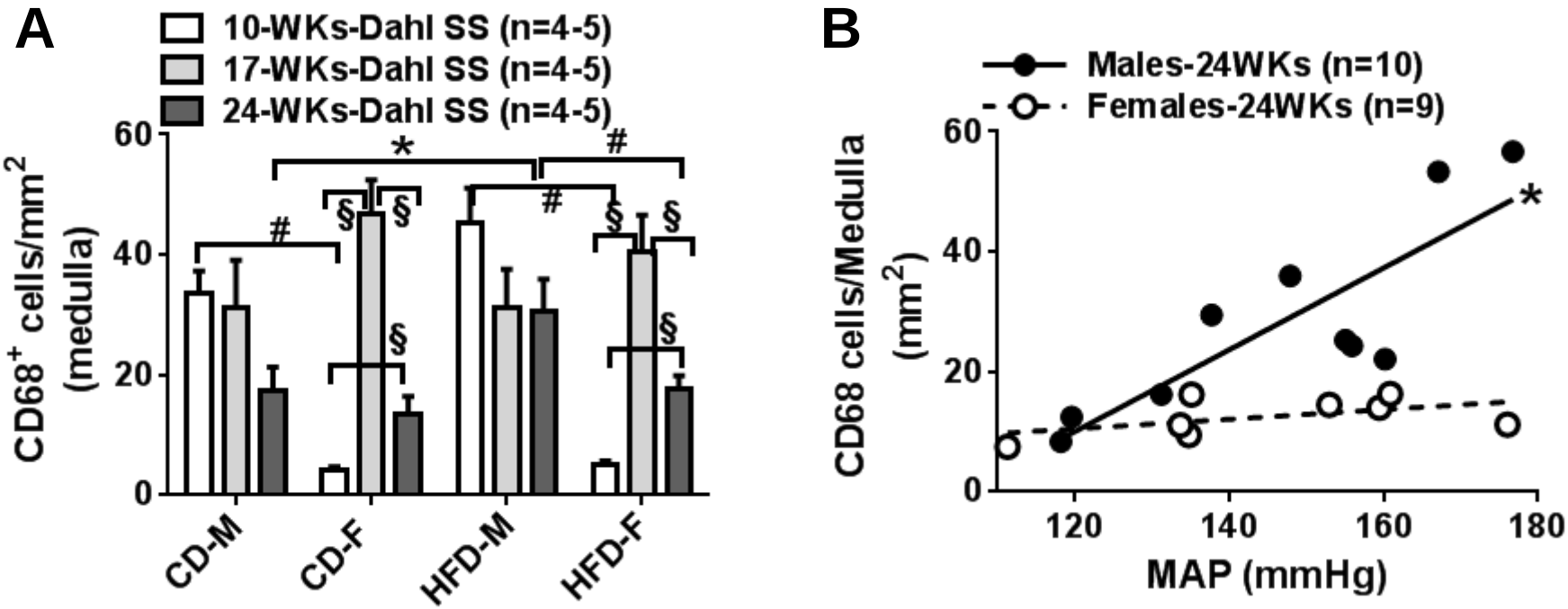
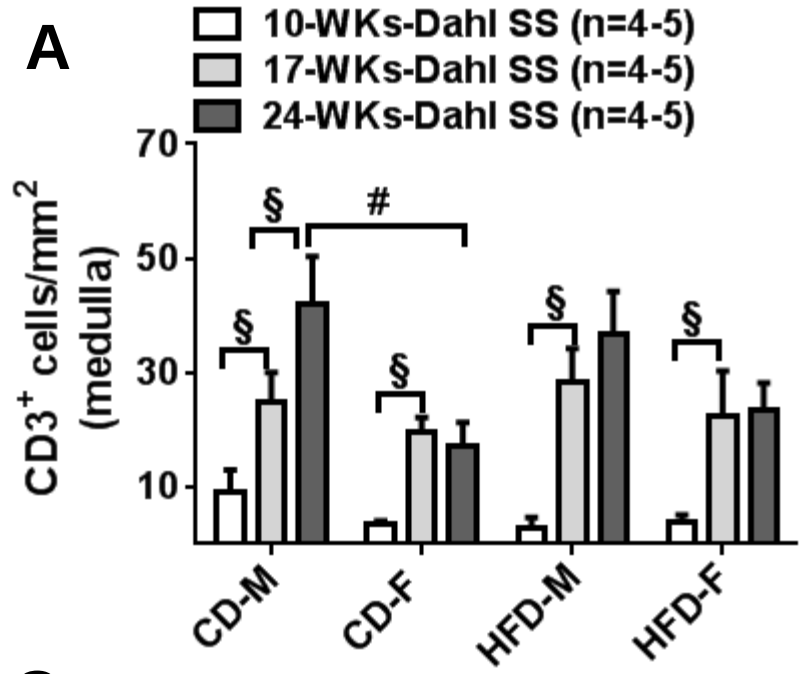
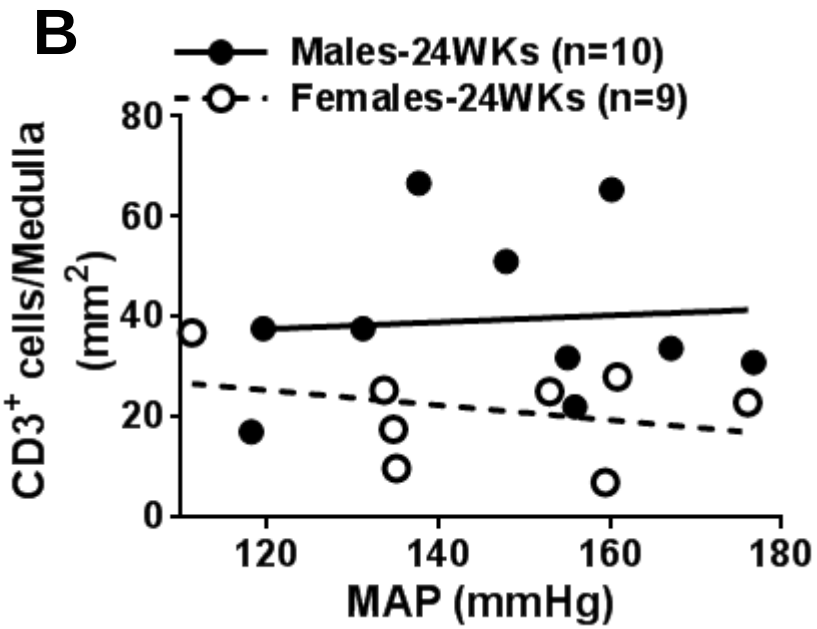


Fig S4: A. Comparisons of medullary macrophages in CD and HFD males and females Dahl SS rats at 10 (10WKs), 17 (17WKs) and 24 weeks (24WKs) of feeding. B. Correlations between blood pressure and medullary $CD68^+$ infiltrations in male and female Dahl SS rats at 24 weeks of feeding.





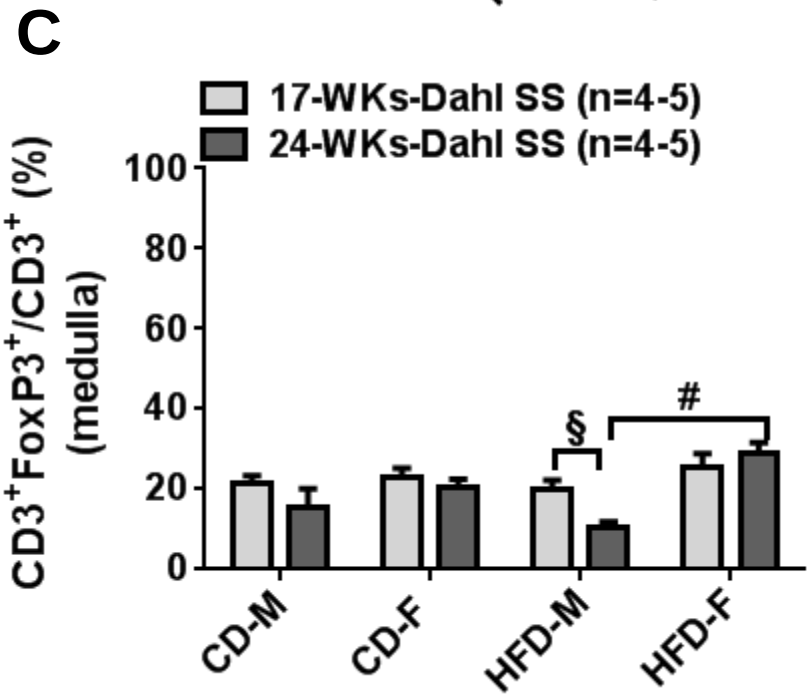


Fig S5: A. Comparisons of medullary T cells in CD and HFD males and females Dahl SS rats at 10 (10WKs), 17 (17WKs) and 24 weeks (24WKs) of feeding. B. Correlations between blood pressure and medullary $CD3^+$ infiltrations in male and female Dahl SS rats at 24 weeks of feeding. C. Correlations between blood pressure and medullary $CD3^+$ infiltrations in male and female Dahl SS rats at 24 weeks of feeding.

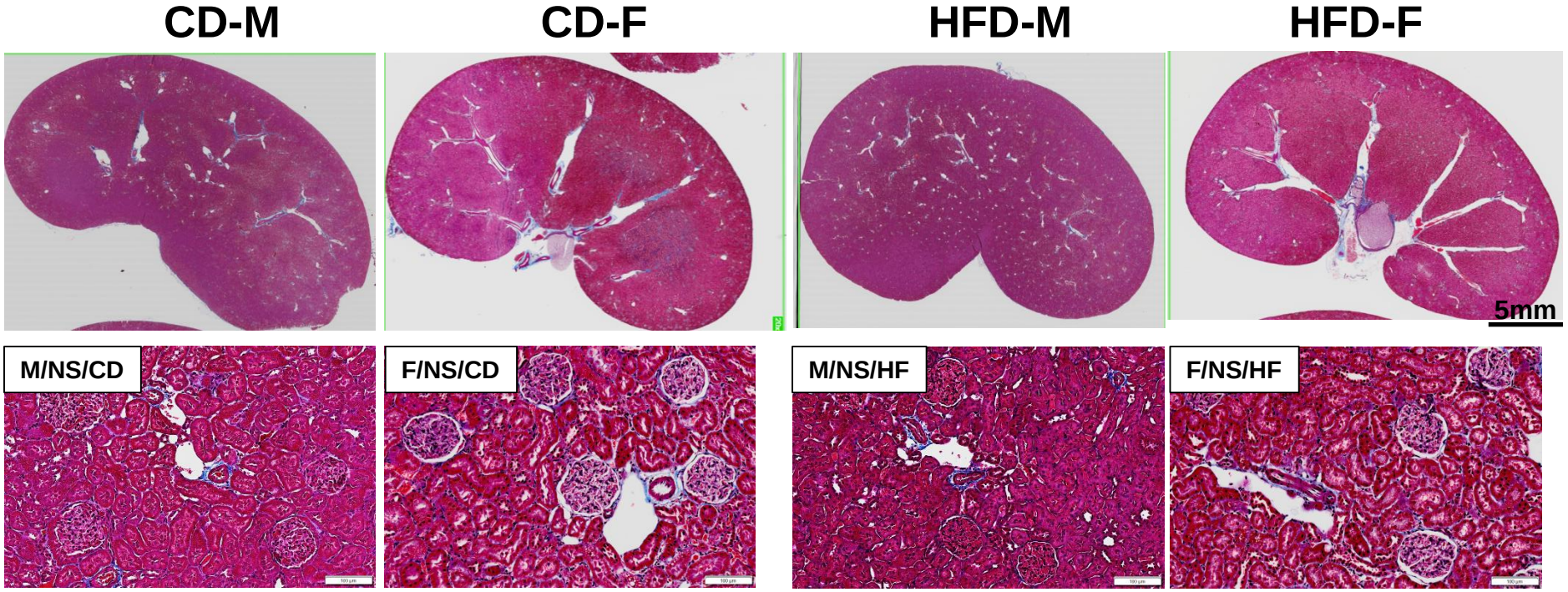


Fig S6: Representative light photomicrographs taken from Masson's Trichrome-stained whole renal sections and higher magnified cortical areas from SD rats at 24 weeks (24WKs) of feeding.