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Complete List of Authors:	Cooper, Timothy; Charles River Laboratories – Contractor Supporting: National Institute of Allergy and Infectious Diseases (NIAID)-Integrated Research Facility, Division of Clinical Research Silva, Kathleen; The Jackson Laboratory, Kennedy, Victoria; The Jackson Laboratory, Alghamdi, Sarah; King Abdullah University of Science and Technology, Computational Bioscience Research Center Hoehndorf, Robert; King Abdullah University of Science and Technology, Computational Bioscience Research Center Sundberg, Beth; The Jackson Laboratory Schofield, Paul; University of Cambridge, 4Dept of Physiology, Development and Neuroscience Sundberg, John; The Jackson Laboratory,
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Hyaline Arteriolosclerosis in 30 Strains of Aged Inbred Mice

Timothy K. Cooper[1*], Kathleen A. Silva[2], Victoria E. Kennedy[2], Sarah Alghamdi[3], Robert Hoehndorf[3], Beth A. Sundberg[2], Paul N. Schofield[2,4], and John P. Sundberg[2]

[1]Departments of Comparative Medicine and Pathology, Penn State Milton S. Hershey Medical Center, Hershey, PA; [2]The Jackson Laboratory, Bar Harbor, ME; [3]Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia, and [4]Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge, UK

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***Corresponding author:** Timothy K Cooper DVM, PhD, Dipl. ACVP, current address:
National Institute of Allergy and Infectious Diseases (NIAID)
Integrated Research Facility
Division of Clinical Research
8200 Research Plaza – Fort Detrick
Frederick, MD 21702

Email: timothy.cooper@nih.gov
Phone: (240) 236-9240
FAX: (301) 631-7389

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Abstract

During a screen for vascular phenotypes in aged laboratory mice, a unique discrete phenotype of hyaline arteriolosclerosis of the intertubular arteries and arterioles of the testes was identified in several inbred strains. Lesions were limited to the testes, and did not occur as part of any renal, systemic, or pulmonary arteriopathy or vasculitis phenotype. There was no evidence of systemic or pulmonary hypertension, and lesions did not occur in female ovaries. Frequency was highest in males of the SM/J (27/30, 90%) and WSB/EiJ (19/26, 73%) strains, aged 383 to 847 days. Lesions were sporadically present in males from several other inbred strains at a much lower (<20%) frequency. The risk of testicular hyaline arteriolosclerosis is at least partially underpinned by a genetic predisposition which is not associated with other vascular lesions (including vasculitis), separating out the etiology of this form and site of arteriolosclerosis from other related conditions that often co-occur in other strains of mice and in humans. Because of their genetic uniformity and controlled dietary and environmental conditions, mice are an excellent model to dissect the pathogenesis of human disease conditions. In this study a discrete genetically driven phenotype of testicular hyaline arteriolosclerosis in aging mice was identified. These observations open the possibility of identifying the underlying genetic variant(s) associated with the predisposition and therefore allowing future interrogation of the pathogenesis of this condition.

Introduction

Arteriolosclerosis, a small arterial or arteriolar subtype of arteriosclerosis, describes thickening of arterial walls with luminal occlusion resulting in loss of elasticity.[10,11,19,28] Hyaline arteriolosclerosis (HAS) specifically describes expansion of the subintima/media by abundant glassy, eosinophilic, amorphous, proteinaceous material with effacement of normal structure. Extreme narrowing can cause ischemia. Although most commonly associated with renal (and glomerular) disease in humans with hypertension or diabetes mellitus, it can also be seen as an age-related change in normotensive individuals, most commonly in the spleen, pancreas, and adrenal, with relative sparing of the kidneys.[19] Impaired blood pressure homeostasis has been suggested as the underlying mechanism for the lesion regardless of etiology.[13] Immunofluorescence studies of frozen renal biopsies have shown that the hyaline material consists of inactivated complement 3b (iC3b) bound to hyaluronic acid, with variable IgM and other complement components thought to arise from new antigens in the iC3b.[11]

Arteriolosclerosis has rarely been reported as an age-related finding in mice.[26] In one study, arteriolosclerosis was identified in 8% and 21% of aging virgin female BALB/cAnNBdf and RFM/Un mice, respectively.[9] Similar to humans, lesions occurred most commonly in the spleen, kidney, and uterus and less commonly in the heart, pancreas, and intestine. Clapp reported a similar distribution of hyaline arteriolosclerosis in 14.1% of aged female RF/Un mice.[8] Interestingly, in figure 206 of that monograph, hyaline arteriolosclerosis was described in the intertubular arteriole (“spermatic artery”) of a single male mouse from a different study, with no further comment. Maita *et al*.

describe an infrequent syndrome of “systemic arteritis” consisting of “marked thickening of the tunica media with a considerable amount of eosinophilic deposits” in large cohort of outbred ICR Crj:CD-1 mice.[18] Lesions affected small to medium arteries, with frequent thrombosis and mild leukocytic infiltration. In addition to the ovary, lesions were observed in uterus, kidney, and heart. Mullink and Haneveld included medial hyalinosis in a wide spectrum of arterial lesions observed in spontaneously hypertensive mice, but little detail is reported.[20] Isolated testicular hyaline arteriolosclerosis has not previously been reported as a phenotype in aging mice or any other animal species.

During a screen for vascular phenotypes in aged laboratory mice, a new discrete phenotype of hyaline arteriolosclerosis of the intertubular arteries and arterioles of the testes was identified in several inbred strains. This investigation describes the frequency and severity by strain of testicular hyaline arteriolosclerosis in aging mice of 30 inbred and wild-derived mouse strains.

Materials and Methods

Mice. The following 30 strains of inbred and wild-derived mice were used in a large-scale aging study[32,35] and, as part of a detailed histopathological analysis, vessels were examined for vascular phenotypes: 129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBRT^{+}tf/J, BUB/BnJ, C3H/HeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, CBA/J, DBA/2J, FVB/NJ, KK/HIJ, LP/J, MRL/MpJ, NOD.B10Sn-H2^{b}/J (NOD; a congenic strain with the NOD genetic background but with a histocompatibility locus from a diabetes-resistant strain), NON/ShiLtJ, NZO/HlLtJ, NZW/LacJ, P/J, PL/J, PWD/PhJ, RIIIS/J, SJL/J, SM/J, SWR/J, and WSB/EiJ. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 weeks of age. Mice were divided into 3 groups. The longitudinal

study (65 females and 35 males; 555-985 days of age) maintained mice until they became morbid or died naturally (i.e., death related to age). Two groups of mice were used in cross-sectional studies to define onset of lesions at defined ages. Mice were euthanized and studied at approximately 12 (372 – 418 days) and 20 (606 - 663 days) months of age, respectively. Cross-sectional and longitudinal study groups were set-up in parallel. The cross-sectional groups and moribund mice were euthanized by CO_2 asphyxiation using methods approved by the American Veterinary Medical Association and complete necropsies were performed.[31] The mouse rooms were maintained on a 12 hr light/12 hr dark cycle and at an ambient temperature of 21-23 °C. Mice of the same sex (4 per cage) were housed in duplex polycarbonate cages (31 x 31 x 214 cm) on pressurized individually ventilated mouse racks (Thoran Caging System; Hazleton, PA) with a high efficiency particulate air-filtered supply and exhaust. Mice were allowed *ad libitum* access to acidified water (pH 2.8 - 3.2) and fed pellets containing 6% fat (LabDiet 5K52, PMI Nutritional International, Bentwood, MO). Regular monitoring for viruses, bacteria, parasites, and microsporidium showed that the colonies were free of any infestation (http://jaxmice.jax.org/genetichealth/index.html). All protocols were reviewed and approved by The Jackson Laboratory Animal Care and Use Committee.

Tissue Fixation and Preparation. Complete necropsies were performed at the time of euthanasia.[31] Tissues were collected, fixed in Fekete's acid-alcohol-formalin solution overnight, and stored in 70% ethanol until processing. Bones were decalcified overnight in Cal-Ex (Fisher, Pittsburgh, PA) and briefly rinsed in water before trimming. Tissues were processed routinely for histology, embedded in paraffin, cut into 6 µm sections, and

stained with hematoxylin and eosin (HE). Additional serial sections were stained with periodic acid Schiff (PAS), Masson's trichrome, and Movat's pentachrome stains.

Histopathologic analyses. All slides were initially reviewed by the same experienced, ACVP diplomate veterinary pathologist (JPS), and the vascular lesions re-evaluated by a second diplomate veterinary pathologist (TKC). Physiological phenotyping data utilizing the International Knockout Mouse Project protocols were generated from this same group of mouse strains and are freely accessible online through the Mouse Phenome Database (MPD, http://phenome.jax.org).[2,3] Vascular lesions were coded to the MPATH and MA ontologies as previously described,[29,33] and anatomical location and pathological diagnosis combined into the precomposed PAM ontology which classifies lesions from MPATH by anatomical site using the MA ontology.[1] Overrepresentation was calculated using the Ontofunc and Func tools[14] as described in Alghamdi *et al*.[1] We performed a hypergeometric test to establish the strains in which vascular lesions are overrepresented. The p value obtained indicates which strains and sex have disproportionately frequent vascular lesions of all types with respect to all the strains examined.

The frequency of HAS lesions was defined as the number of mice carrying diagnosed lesions by strain and sex. Lesions were also characterized by severity scores for each mouse (scores: 0 – normal; 1 - minimal; 2 - mild; 3 - moderate; 4 - severe). Average scores of all affected mice per strain were obtained.

Results

Description of Phenotype

Lesions were essentially limited to the small parenchymal (intertubular) arteries and arterioles of the testes (Figs. 1-3). Lesion severity was semi-quantitatively evaluated on the basis of number and size of vessels affected as well as degree of vascular lesions. In minimal lesions, rare small arteriolar walls were expanded by eccentric medial drops of brightly eosinophilic hyaline proteinaceous material. These droplets coalesced to expand the wall and impinged on the lumen in mild and moderate lesions, with increasing numbers of vessels affected. In severe lesions, multiple arterioles and small arteries were markedly expanded by abundant medial hyaline that compressed or obliterated the lumina. Lesions were segmental within arteries, and were particularly prominent at arterial branch points. There were various degrees of mild to moderate concentric adventitial fibroplasia or fibrosis (Fig. 4), with occasional adventitial infiltrates of low to rarely moderate numbers of plasma cells, macrophages, and lymphocytes. Rarely mice had overt leukocytic infiltration of intima/media (macrophages, viable and degenerate neutrophils, pyknotic nuclei).

Lesions in SM/J mice predominantly affected small arteries and, to a lesser extent, arterioles. By contrast, lesions in WSB/EiJ mice were essentially limited to arterioles (Fig. 2). Lesions were not present in any other organs, including kidney, pancreas, and spleen. No other vascular lesions (e.g. polyarteritis nodosa (PAN), medial mineralization, hyaline glomerulopathy, atherosclerosis, etc.) were present in these mice. In two SM/J mice there was mild to moderate hyaline arteriolosclerosis of a few renal arcuate arteries. One WSB/EiJ mouse also had focal hyaline arteriolosclerosis in the splenic red pulp. Other than these exceptions, lesions were not present in any other organs/sites.

Hyaline material was weakly to intensely PAS positive (Fig. 5). In a few two year old SM/J mice there were scattered lesions consistent with hyperplastic arteriolosclerosis, with concentric layers of plump hypertrophic smooth muscle cells in a loose (onionskin-like) matrix, highlighted by PAS staining (Fig. 6). Hyaline material was intensely red with Masson's trichrome (Fig. 7) and Movat's pentachrome, and there was occasional adventitial fibrosis (Fig. 8). By Movat's pentachrome, there was fragmentation or loss of the internal elastic lamina, with accumulation of the hyaline material in the subendothelial space. By Masson's trichrome, material varied from intensely bright red (fibrinoid) to blue and fibrillar (fibrosis).

Frequency and Severity of Lesions by Strain

Hyaline arteriolosclerosis lesions were not noted in females of any strain in any organ. Overall lesion frequency in males was highest in the SM/J (0.9) and WSB/EiJ (0.73) strains (Table 1 and Fig. 9). Lesions were more severe in SM/J than in WSB/EiJ, and in both strains the lesion severity increased with age from 12 to 20 months (2.6 to 3.77 and 1.33 to 2.09, respectively). HAS lesions appeared sporadically in individual males of other strains, including 129S1/SvImJ (5/42), A/J (1/32), BUB/BnJ (2/22), DBA/2J (1/24), and FVB/NJ (1/26). No lesions were present in mice from BALB/cByJ (n=31 males), BTBRT^{+} tf/J (23), C3H/HeJ (29), C57BL/10J (32), C57BL/6J (39), C57BLKS/J (44), C57L/J (32), KK/HIJ (30), LP/J (37), MRL/MpJ (30), NON/ShiLtJ (28), NZO/HlLtJ (12), NZW/LacJ (27), PL/J (19), PWD/PhJ (25), RIIIS/J (32), SJL/J (11), or SWR/J (19) strains. Overrepresentation analysis of complete screening data, coded using the Mouse Pathology (MPATH) and Mouse Anatomy (MA) ontologies,[33] excluding testicular HAS, showed an overall excess of vasculitis (polyarteritis nodosa) in females of BUB/BnJ and

in males of NZO/H1LtJ (p=0.008, p=0.0006 respectively). Compared with the distribution of HAS across these strains, this suggests that factors predisposing to the testicular lesions in SM/J and WSB/EiJ, do not seem to produce an overall tendency to vasculitis/vascular lesions in other organs or in other strains, indicating distinct genetic predisposition for this lesion. Lesions were present at high or low frequency in strains from all mouse family tree groups except Group 3 (Japanese and New Zealand inbred strains).

Discussion

The frequency of arteriolosclerosis, often subsumed under the general condition of small vessel disease (SVD),[4] or more accurately hyaline arteriolar sclerosis, in inbred strains of laboratory mice has not been systematically reported previously. Here we show that the lesion is almost uniquely present in the testis of two inbred strains of mice, and increases in frequency as these male mice age. Hyaline arteriolosclerosis has not been reported previously in the intra-testicular arterial system of rodents,[7] as discussed above, but age-associated testicular HAS occurs in man.[30] As with all types of vascular lesions, extra-testicular HAS seem to have a more uniform distribution between strains and much lower frequency; the resulting implication is that its localized occurrence in the testicular vasculature of SM/J and WSB/EiJ mice has a specific genetic component.

SM/J mice were developed by MacArthur from seven stocks and were selected for small body size.[17] SM/J mice are susceptible to atherosclerosis when fed a high-fat diet, but maintain a normal high-density lipoprotein level [http://www.informatics.jax.org/external/festing/mouse/docs/SM.shtml]. Interestingly,

SM/J mice are reported to be difficult breeders. The relationship between the observed arterial disease and poor reproductive performance remains unexplored. However, there was no direct relationship between observed degeneration of the seminiferous tubules and arterial lesions present in examined mice. WSB/EiJ mice are particularly long lived, but SM/J males are regarded as having an intermediate lifespan (median lifespan 783d vs 871d for WSB/EiJ), mitigating against any argument that the lesions are simply age dependent.[35] The SM/J strain is related to the C.C. Little's DBA and related strains (Group 6) derived from *Mus musculus domesticus*.[25] There was a low frequency of lesions in the only other Group 6 strains examined, DBA/2J and P/J.

Watkins star line B (WSB/EiJ) mice were derived from wild *M. m. domesticus* mice trapped on Maryland's Eastern Shore by Michael Potter in 1976.[27] Notably WSB/EiJ contains an allele of *R2d2* which is subject to meiotic drive favoring its transmission, and shows no evidence of introgression from other subspecies.[5] SM/J (Group 6) and WSB/EiJ (Group 7, wild-derived strains) are not closely-related strains, suggesting that any shared genetic predisposition to HAS either was present in common ancestral *M. m. domesticus* mouse populations and subsequently lost from or otherwise suppressed in related strains, or more likely arose spontaneously and independently in these two lineages and became fixed through inbreeding.[25,34] Notably, testicular HAS lesions were absent from the PWD/PhJ strain, the only other member of Group 7 examined. Phylogenetic relationships suggest that the closely related LEWES/EiJ might be informative as it is of all the wild derived strains most closely related to WSB/EiJ.[25] The presence of low frequency of lesions and negative findings within multiple strain Groups, including within two closely related strains (e.g. C57L/J

and C57BR/cdJ, FVBN/J and SWR/J) may also argue for a multigenic mode of inheritance.

Human studies have indicated several genes that may be involved in predisposition to HAS, mainly in circumstances of normal or accelerated aging. CNS degenerative disease is often associated with vascular lesions. *ABCC9* variants have been implicated in hippocampal sclerosis;[15,21-23] *HTRA1* variants are associated with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), though in this case HAS was also described in extra-cerebral, visceral sites;[16] and *LMNA* is implicated in Hutchinson-Gilford Progeria syndrome.[24] A study of stroke predisposition associated a diabetes risk allele within *JAZF1* with arteriolosclerosis,[6] and a variant in *GNB3* is implicated in radial vasculature hypertrophy.[12] Of these candidates only *Lmna* and *Abcc9* have vascular phenotypes in null mice (listed as abnormal vascular smooth muscle physiology; Mouse Genome Informatics, accessed 3 January 2019) and none specifically report HAS, testicular or otherwise (Mouse Genome Informatics, accessed 3 January 2019). Mice with a null allele of *Jazf1* have very recently been reported, but the only abnormal phenotype described is reduced circulating fasting insulin levels. There is no reported expression in the vasculature or the gonad of either sex (http://www.mousephenotype.org/data/genes/MGI:2141450#section-associations). Although this discrete phenotype of testicular HAS would be an ideal candidate for genome-wide association studies, current SNP coverage density of the SM/J strain is insufficient to robustly identify candidate genes. An in-depth interrogation of the genetics underpinning this phenotype therefore awaits better sequencing of this strain.

Because of their well-defined genetics, including the availability of inbred strains, combined with the accessibility of the testes to manipulation or even unilateral excision, this mouse model presents a unique opportunity to study the genetics and pathogenesis of age-related arteriolosclerosis, as well as the possible interaction with male infertility. Additional larger future studies, including younger mice, may further refine the pathogenesis of this phenotype.

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Table 1. Frequency and severity of testicular arteriolosclerosis by age and strain. Number in parentheses after group is total number of male mice in that group. Mean arteriolosclerosis score is the average disease severity score of all mice (including normal, score 0), for each strain.

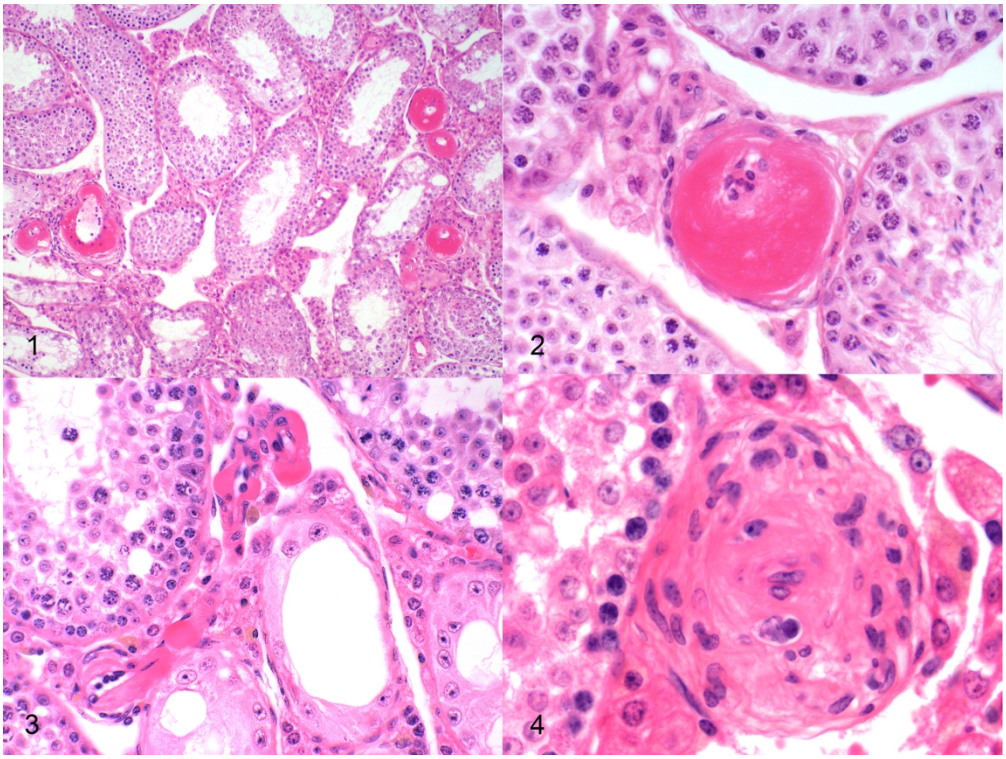
Number in parentheses after mean arteriolosclerosis severity score is average score in affected mice only.

Figures 1-4. Arteriolosclerosis, testes, mice. Figure 1. Hyaline material expands the media of multiple medium and small intertubular arteries in a 20 month old SM/J mouse. Unaffected arteries are present, demonstrating the segmental nature of the lesions. Hematoxylin & eosin (HE). Figure 2. Abundant hyaline eosinophilic material expands the media and compresses the lumen of a small intertubular artery of a 20 month old SM/J. HE. Figure 3. Arteriolosclerosis of the arterioles in a 20 month old WSB/EiJ. HE. Figure 4. Chronic lesion with florid adventitial fibrosis in a 20 month old SM/J mouse. HE.

Figures 5-8. Arteriolosclerosis, testes, mice. Figure 5. Periodic acid Schiff (PAS) positive hyaline material expands the arterial media in a 20 month old SM/J mouse. PAS. Figure 6. Hyperplastic smooth muscle in the media of a small testicular artery of a 20 month old SM/J mouse. PAS. Figure 7. Abundant medial fibrinoid material (bright red) admixes with fibrillar collagen (blue) in a 12 month old SM/J mouse.. Masson's trichome. Figure 8. Different arterial segment from the same mouse showing mature diffuse medial and adventitial fibrosis and demonstrating the variable nature of lesions within adjacent vessels. Masson's trichrome.

Figure 9. Testicular arteriolosclerosis by mouse strain. Adapted from Petkov *et al.* Used with permission. The phenotype of testicular HAS is present with varying frequency in multiple

genetically divergent and distinct inbred mouse strains. Red boxes delineate strains with high frequency; blue boxes for strains with low frequency, and grey boxes for strains negative for the phenotype. The PWD/PhJ strain (negative, not shown) is in group 7. The RF strain in Group 1 (turquoise) was previously described to develop these lesions, but no frequency was reported.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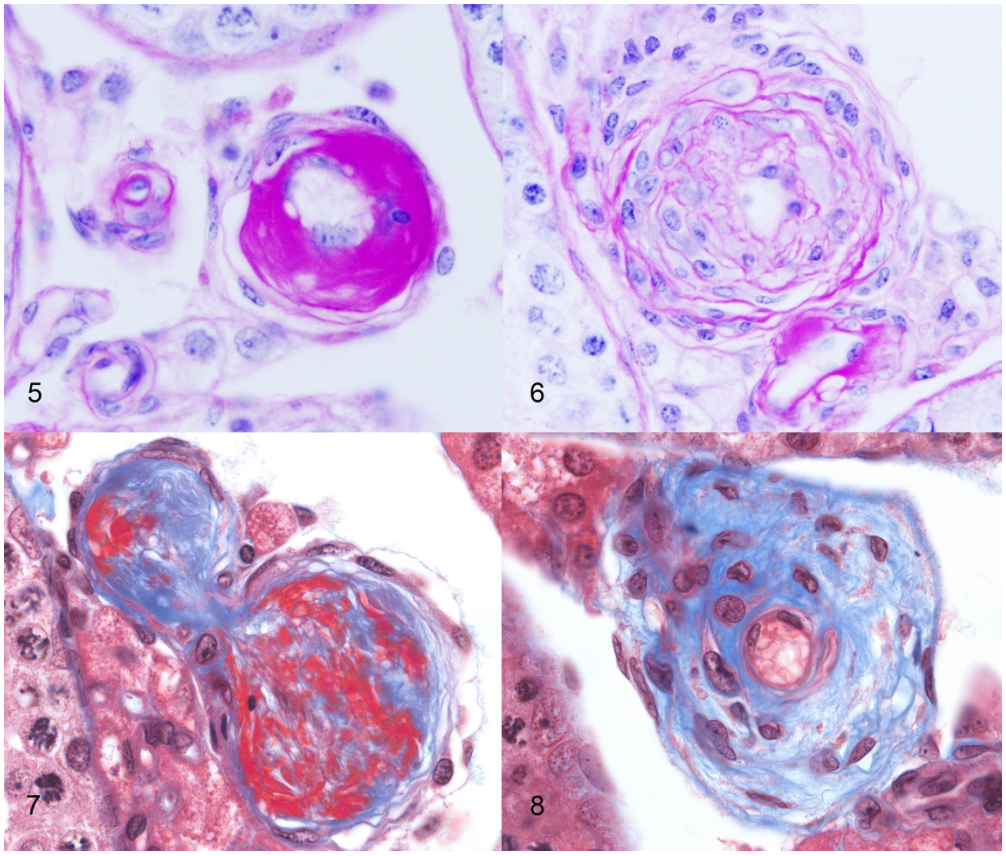
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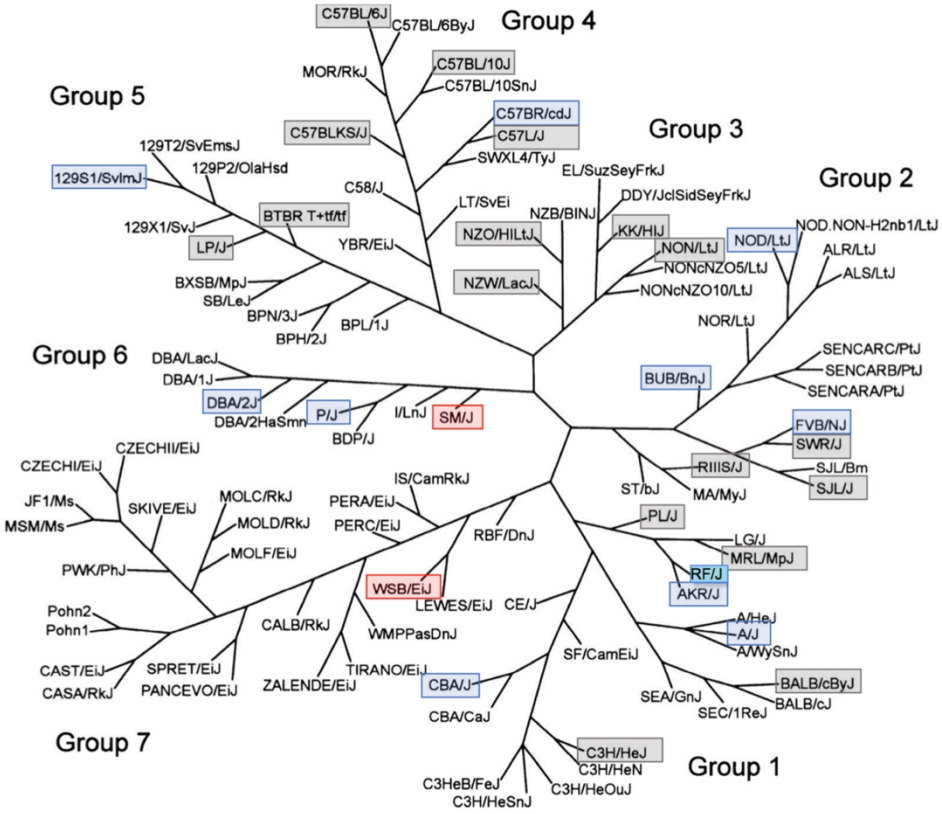

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Table 1. Frequency and severity of testicular arteriolosclerosis by age and strain.

Inbred Strain	Overall Frequency	Study Group	Group Frequency	Mean Arteriolosclerosis score
SM/J	0.9	12 months (13)	0.85	2.15 (2.6)
		20 months (13)	1	3.77
		Longitudinal (4)	0.75	1.75 (2.33)
WSB/EiJ	0.73	12 months (13)	0.54	0.77 (1.33)
		20 months (11)	1	2.09
		Longitudinal (2)	0.5	1.5 (3)
129S1/SvImJ	0.12	12 months (15)	0	0
		20 months (15)	0.13	0.27 (2)
		Longitudinal (12)	0.25	0.5 (2)
C57BR/cdJ	0.09	12 months (16)	0	0
		20 months (15)	0.13	0.2 (2)
		Longitudinal (3)	0.33	1.3 (2.5)
BUB/BnJ	0.17	12 months (10)	0.2	0.3 (1.5)
		20 months (2)	0	0
A/J	0.03	12 months (13)	0	0
		20 months (9)	0.07	0.13 (2)
		Longitudinal (10)	0	0
AKR/J	0.08	12 months (10)	0.1	0.1 (1)
		Longitudinal (2)	0	0
CBA/J	0.05	12 months (14)	0	0
		20 months (11)	0.09	0.18 (2)
		Longitudinal (7)	0	0
DBA/2J	0.04	12 months (11)	0	0
		20 months (6)	0.17	0.17 (1)

		Longitudinal (7)	0	0
FVB/NJ	0.04	12 months (15)	0	0
		20 months (7)	0.14	0.29 (2)
		Longitudinal (4)	0	0
P/J	0.11	12 months (3)	0	0
		20 months (6)	0.17	0.67 (4)
NOD.B10Sn-H2^{b}/J	0.05	12 months (12)	0.08	0.08 (1)
		20 months (9)	0	0
		Longitudinal (1)	0	0

Hyaline Arteriolosclerosis in 30 Strains of Aged Inbred Mice

Timothy K. Cooper[1*], Kathleen A. Silva[2], Victoria E. Kennedy[2], Sarah Alghamdi[3], Robert Hoehndorf[3], Beth A. Sundberg[2], Paul N. Schofield[2,4], and John P. Sundberg[2]

[1]Departments of Comparative Medicine and Pathology, Penn State Milton S. Hershey Medical Center, Hershey, PA; [2]The Jackson Laboratory, Bar Harbor, ME; [3]Computational Bioscience Research Center, King Abdullah University of Science and Technology, Thuwal, Kingdom of Saudi Arabia, and [4]Department of Physiology Development and Neuroscience, University of Cambridge, Cambridge, UK

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***Corresponding author:** Timothy K Cooper DVM, PhD, Dipl. ACVP, current address:
National Institute of Allergy and Infectious Diseases (NIAID)
Integrated Research Facility
Division of Clinical Research
8200 Research Plaza – Fort Detrick
Frederick, MD 21702

Email: timothy.cooper@nih.gov
Phone: (240) 236-9240
FAX: (301) 631-7389

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Abstract

During a screen for vascular phenotypes in aged laboratory mice, a unique discrete phenotype of hyaline arteriolosclerosis of the intertubular arteries and arterioles of the testes was identified in several inbred strains. Lesions were limited to the testes, and did not occur as part of any renal, systemic, or pulmonary arteriopathy or vasculitis phenotype. There was no evidence of systemic or pulmonary hypertension, and lesions did not occur in female ovaries. Frequency was highest in males of the SM/J (27/30, 90%) and WSB/EiJ (19/26, 73%) strains, aged 383 to 847 days. Lesions were sporadically present in males from several other inbred strains at a much lower (<20%) frequency. The risk of testicular hyaline arteriolosclerosis is at least partially underpinned by a genetic predisposition which is not associated with other vascular lesions (including vasculitis), separating out the etiology of this form and site of arteriolosclerosis from other related conditions that often co-occur in other strains of mice and in humans. Because of their genetic uniformity and controlled dietary and environmental conditions, mice are an excellent model to dissect the pathogenesis of human disease conditions. In this study a discrete genetically driven phenotype of testicular hyaline arteriolosclerosis in aging mice was identified. These observations open the possibility of identifying the underlying genetic variant(s) associated with the predisposition and therefore allowing future interrogation of the pathogenesis of this condition.

Introduction

Arteriolosclerosis, a small arterial or arteriolar subtype of arteriosclerosis, describes thickening of arterial walls with luminal occlusion resulting in loss of elasticity.[10,11,19,28] Hyaline arteriolosclerosis (HAS) specifically describes expansion of the subintima/media by abundant glassy, eosinophilic, amorphous, proteinaceous material with effacement of normal structure. Extreme narrowing can cause ischemia. Although most commonly associated with renal (and glomerular) disease in humans with hypertension or diabetes mellitus, it can also be seen as an age-related change in normotensive individuals, most commonly in the spleen, pancreas, and adrenal, with relative sparing of the kidneys.[19] Impaired blood pressure homeostasis has been suggested as the underlying mechanism for the lesion regardless of etiology.[13] Immunofluorescence studies of frozen renal biopsies have shown that the hyaline material consists of inactivated complement 3b (iC3b) bound to hyaluronic acid, with variable IgM and other complement components thought to arise from new antigens in the iC3b.[11]

Arteriolosclerosis has rarely been reported as an age-related finding in mice.[26] In one study, arteriolosclerosis was identified in 8% and 21% of aging virgin female BALB/cAnNBdf and RFM/Un mice, respectively.[9] Similar to humans, lesions occurred most commonly in the spleen, kidney, and uterus and less commonly in the heart, pancreas, and intestine. Clapp reported a similar distribution of hyaline arteriolosclerosis in 14.1% of aged female RF/Un mice.[8] Interestingly, in figure 206 of that monograph, hyaline arteriolosclerosis was described in the intertubular arteriole (“spermatic artery”) of a single male mouse from a different study, with no further comment ~~(figure 206)~~. Maita

et al. describe an infrequent syndrome of "systemic arteritis" consisting of "marked thickening of the tunica media with a considerable amount of eosinophilic deposits" in large cohort of outbred ICR Crj:CD-1 mice.[18] Lesions affected small to medium arteries, with frequent thrombosis and mild leukocytic infiltration. In addition to the ovary, lesions were observed in uterus, kidney, and heart. Mullink and Haneveld included medial hyalinosis in a wide spectrum of arterial lesions observed in spontaneously hypertensive mice, but little detail is reported.[20] Isolated testicular hyaline arteriolosclerosis has not previously been reported as a phenotype in aging mice or any other animal species.

During a screen for vascular phenotypes in aged laboratory mice, a new discrete phenotype of hyaline arteriolosclerosis of the intertubular arteries and arterioles of the testes was identified in several inbred strains. This investigation describes the frequency and severity by strain of testicular hyaline arteriolosclerosis in aging mice of 30 inbred and wild-derived mouse strains.

Materials and Methods

Mice. The following 30 strains of inbred and wild-derived mice were used in a large-scale aging study[32,35] and, as part of a detailed histopathological analysis, vessels were examined for vascular phenotypes: 129S1/SvImJ, A/J, AKR/J, BALB/cByJ, BTBRT^{+}tf/J, BUB/BnJ, C3H/HeJ, C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ, C57L/J, CBA/J, DBA/2J, FVB/NJ, KK/HIJ, LP/J, MRL/MpJ, NOD.B10Sn-H2^{b}/J (NOD; a congenic strain with the NOD genetic background but with a histocompatibility locus from a diabetes-resistant strain), NON/ShiLtJ, NZO/HlLtJ, NZW/LacJ, P/J, PL/J, PWD/PhJ, RIIIS/J, SJL/J, SM/J, SWR/J, and WSB/EiJ. All mice were obtained from The Jackson Laboratory (Bar Harbor, ME) at 6 to 8 weeks of age. Mice were divided into 3 groups. The longitudinal

study (65 females and 35 males; 555-985 days of age) maintained mice until they became morbid or died naturally (i.e., death related to age). Two groups of mice were used in cross-sectional studies to define onset of lesions at defined ages. Mice were euthanized and studied at approximately 12 (372 – 418 days) and 20 (606 - 663 days) months of age, respectively. Cross-sectional and longitudinal study groups were set-up in parallel. The cross-sectional groups and moribund mice were euthanized by CO_2 asphyxiation using methods approved by the American Veterinary Medical Association and complete necropsies were performed.[31] The mouse rooms were maintained on a 12 hr light/12 hr dark cycle and at an ambient temperature of 21-23 °C. Mice of the same sex (4 per cage) were housed in duplex polycarbonate cages (31 x 31 x 214 cm) on pressurized individually ventilated mouse racks (Thoran Caging System; Hazleton, PA) with a high efficiency particulate air-filtered supply and exhaust. Mice were allowed *ad libitum* access to acidified water (pH 2.8 - 3.2) and fed pellets containing 6% fat (LabDiet 5K52, PMI Nutritional International, Bentwood, MO). Regular monitoring for viruses, bacteria, parasites, and microsporidium showed that the colonies were free of any infestation (http://jaxmice.jax.org/genetichealth/index.html). All protocols were reviewed and approved by The Jackson Laboratory Animal Care and Use Committee.

Tissue Fixation and Preparation. Complete necropsies were performed at the time of euthanasia.[31] Tissues were collected, fixed in Fekete's acid-alcohol-formalin solution overnight, and stored in 70% ethanol until processing. Bones were decalcified overnight in Cal-Ex (Fisher, Pittsburgh, PA) and briefly rinsed in water before trimming. Tissues were processed routinely for histology, embedded in paraffin, cut into 6 µm sections, and

stained with hematoxylin and eosin (HE). Additional serial sections were stained with periodic acid Schiff (PAS), Masson's trichrome, and Movat's pentachrome stains.

Histopathologic analyses. All slides were initially reviewed by the same experienced, ACVP diplomate veterinary pathologist (JPS), and the vascular lesions re-evaluated by a second diplomate veterinary pathologist (TKC). Physiological phenotyping data utilizing the International Knockout Mouse Project protocols were generated from this same group of mouse strains and are freely accessible online through the Mouse Phenome Database (MPD, http://phenome.jax.org).[2,3] Vascular lesions were coded to the MPATH and MA ontologies as previously described,[29,33] and anatomical location and pathological diagnosis combined into the precomposed PAM ontology which classifies lesions from MPATH by anatomical site using the MA ontology.[1] Overrepresentation was calculated using the Ontofunc and Func tools[14] as described in Alghamdi *et al*.[1] We performed a hypergeometric test to establish the strains in which vascular lesions are overrepresented. The p value obtained indicates which strains and sex have disproportionately frequent vascular lesions of all types with respect to all the strains examined.

The frequency of HAS lesions was defined as the number of mice carrying diagnosed lesions by strain and sex. Lesions were also characterized by severity scores for each mouse (scores: 0 – normal; 1 - minimal; 2 - mild; 3 - moderate; 4 - severe). Average scores of all affected mice per strain were obtained.

Results

Description of Phenotype

Lesions were essentially limited to the small parenchymal (intertubular) arteries and arterioles of the testes (Figs. 1-3). Lesion severity was semi-quantitatively evaluated on the basis of number and size of vessels affected as well as degree of vascular lesions. In minimal lesions, rare small arteriolar walls were expanded by eccentric medial drops of brightly eosinophilic hyaline proteinaceous material. These droplets coalesced to ~~markedly~~ expand the wall and ~~often compress~~impinged on the lumen in mild and moderate lesions, with increasing numbers of vessels affected. In severe lesions, multiple arterioles and small arteries were markedly expanded by abundant medial hyaline that compressed or obliterated the lumina. Lesions were segmental within arteries, and were particularly prominent at arterial branch points. There were various degrees of mild to moderate concentric adventitial fibroplasia or fibrosis (Fig. 4), with occasional adventitial infiltrates of low to rarely moderate numbers of plasma cells, macrophages, and lymphocytes. Rarely mice had overt leukocytic infiltration of intima/media (macrophages, viable and degenerate neutrophils, pyknotic nuclei).

Lesions in SM/J mice predominantly affected small arteries and, to a lesser extent, arterioles. By contrast, lesions in WSB/EiJ mice were essentially limited to arterioles (Fig. 2). Lesions were not present in any other organs, including kidney, pancreas, and spleen. No other vascular lesions (e.g. polyarteritis nodosa (PAN), medial mineralization, hyaline glomerulopathy, atherosclerosis, etc.) were present in these mice. In two SM/J mice there was mild to moderate hyaline arteriolosclerosis of a few renal arcuate arteries. One WSB/EiJ mouse also had focal hyaline arteriolosclerosis in the splenic red pulp. Other than these exceptions, lesions were not present in any other organs/sites.

Hyaline material was weakly to intensely PAS positive (Fig. 5). In a few two year old SM/J mice there were scattered lesions consistent with hyperplastic arteriolosclerosis, with concentric layers of plump hypertrophic smooth muscle cells in a loose (onionskin-like) matrix, highlighted by PAS staining (Fig. 6). Hyaline material was intensely red with Masson's trichrome (Fig. 7) and Movat's pentachrome ~~(Fig. 7)~~, and there was occasional adventitial fibrosis (Fig. 8). By Movat's pentachrome, there was fragmentation or loss of the internal elastic lamina, with accumulation of the hyaline material in the subendothelial space. ~~M~~By Masson's trichrome, material varied from intensely bright red (fibrinoid) to ~~pale yellow~~blue and fibrillar (fibrosis).

Frequency and Severity of Lesions by Strain

Hyaline arteriolosclerosis lesions were not noted in females of any strain in any organ. Overall ~~I~~lesion frequency in males was highest in the SM/J (0.9) and WSB/EiJ (0.73) strains~~, and severity increased with age~~ (Table 1 and Fig. 9). Lesions were more severe in SM/J than in WSB/EiJ, and in both strains the lesion severity increased with age from 12 to 20 months (2.6 to 3.77 and 1.33 to 2.09, respectively). HAS lesions appeared sporadically in individual ~~animals~~ males of other strains, including 129S1/SvImJ (5/42), A/J (1/32), BUB/BnJ (2/22), DBA/2J (1/24), and FVB/NJ (1/26). No lesions were present in mice from BALB/cByJ (n=31 males), BTBRT^{+} tf/J (23), C3H/HeJ (29), C57BL/10J (32), C57BL/6J (39), C57BLKS/J (44), C57L/J (32), KK/HlJ (30), LP/J (37), MRL/MpJ (30), NON/ShiLtJ (28), NZO/HlLtJ (12), NZW/LacJ (27), PL/J (19), PWD/PhJ (25), RIIIS/J (32), SJL/J (11), or SWR/J (19) strains. Overrepresentation analysis of complete screening data, coded using the Mouse Pathology (MPATH) and Mouse Anatomy (MA) ontologies,[33] excluding testicular HAS, showed an overall excess of ~~all~~

types of vasculitis (polyarteritis nodosa) in females of BUB/BnJ and in males of NZO/H1LtJ (p=0.008, p=0.0006 respectively). Polyarteritis nodosa was significantly overrepresented in female BUB/BnJ (p=0.02), and male NZO/H1LtJ (p=0.0001). Compared with the distribution of HAS across these strains, this suggests that factors predisposing to the testicular lesions in SM/J and WSB/EiJ, do not seem to produce an overall tendency to vasculitis/vascular lesions in other organs or in other strains, indicating distinct genetic predisposition for this lesion. Lesions were present at high or low frequency in strains from all mouse family tree groups except Group 3 (Japanese and New Zealand inbred strains).

Discussion

The frequency of arteriolosclerosis, often subsumed under the general condition of small vessel disease (SVD),[4] or more accurately hyaline arteriolar sclerosis, in inbred strains of laboratory mice has not been systematically reported previously. Here we show that the lesion is almost uniquely present in the testis of two inbred strains of mice, and increases in frequency as these male mice age. Hyaline arteriolosclerosis has not been reported previously in the intra-testicular arterial system of rodents,[7] as discussed above, but age-associated testicular HAS occurs in man.[30] As with all types of vascular lesions, extra-testicular HAS seem to have a more uniform distribution between strains and much lower frequency; the resulting implication is that its localized occurrence in the testicular vasculature of SM/J and WSB/EiJ mice has a specific genetic component.

SM/J mice were developed by MacArthur from seven stocks and were selected for small body size.[17] SM/J mice are susceptible to atherosclerosis when fed a high-fat

diet, but maintain a normal high-density lipoprotein level [http://www.informatics.jax.org/external/festing/mouse/docs/SM.shtml]. Interestingly, SM/J mice are reported to be difficult breeders. The relationship between the observed arterial disease and poor reproductive performance remains unexplored. However, there was no direct relationship between observed degeneration of the seminiferous tubules and arterial lesions present in examined mice. WSB/EiJ mice are particularly long lived, but SM/J males are regarded as having an intermediate lifespan (median lifespan 783d vs 871d for WSB/EiJ), mitigating against any argument that the lesions are simply age dependent.[35] The SM/J strain is related to the C.C. Little's DBA and related strains (Group 6) derived from *Mus musculus domesticus*.[25] There was a low ~~incidence~~ frequency of lesions in the only other Group 6 strains examined, DBA/2J and P/J.

Watkins star line B (WSB/EiJ) mice were derived from wild *M. m. domesticus* mice trapped on Maryland's Eastern Shore by Michael Potter in 1976.[27] Notably WSB/EiJ contains an allele of *R2d2* which is subject to meiotic drive favoring its transmission, and shows no evidence of introgression from other subspecies.[5] SM/J (Group 6) and WSB/EiJ (Group 7, wild-derived strains) are not closely-related strains, suggesting that any shared genetic predisposition to HAS either was present in common ancestral *M. m. domesticus* mouse populations and subsequently lost from or otherwise suppressed in related strains, or more likely arose spontaneously and independently in these two lineages and became fixed through inbreeding.[25,34] Notably, testicular HAS lesions were absent from the PWD/PhJ strain, the only other member of Group 7 examined. Phylogenetic relationships suggest that the closely related

LEWES/EiJ might be informative as it is of all the wild derived strains most closely related to WSB/EiJ.[25] The presence of low frequency of lesions and negative findings within multiple strain Groups, including within two closely related strains (e.g. C57L/J and C57BR/cdJ, FVBN/J and SWR/J) may also argue for a multigenic mode of inheritance.

Human studies have indicated several genes that may be involved in predisposition to HAS, mainly in circumstances of normal or accelerated aging. CNS degenerative disease is often associated with vascular lesions. *ABCC9* variants have been implicated in hippocampal sclerosis;[15,21-23] *HTRA1* variants are associated with cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL), though in this case HAS was also described in extra-cerebral, visceral sites;[16] and *LMNA* is implicated in Hutchinson-Gilford Progeria syndrome.[24] A study of stroke predisposition associated a diabetes risk allele within *JAZF1* with arteriolosclerosis,[6] and a variant in *GNB3* is implicated in radial vasculature hypertrophy.[12] Of these candidates only *Lmna* and *Abcc9* have vascular phenotypes in null mice (listed as abnormal vascular smooth muscle physiology; Mouse Genome Informatics, accessed 3 January 2019) and none specifically report HAS, testicular or otherwise (Mouse Genome Informatics, accessed 3 January 2019). Mice with a null allele of *Jazf1* have very recently been reported, but the only abnormal phenotype described is reduced circulating fasting insulin levels. There is no reported expression in the vasculature or the gonad of either sex (http://www.mousephenotype.org/data/genes/MGI:2141450#section-associations). Although this discrete phenotype of testicular HAS would be an ideal candidate for

genome-wide association studies, current SNP coverage density of the SM/J strain is insufficient to robustly identify candidate genes. An in-depth interrogation of the genetics underpinning this phenotype therefore awaits better sequencing of this strain.

Because of their well-defined genetics, including the availability of inbred strains, combined with the accessibility of the testes to manipulation or even unilateral excision, this mouse model presents a unique opportunity to study the genetics and pathogenesis of age-related arteriolosclerosis, as well as the possible interaction with male infertility. Additional larger future studies, including younger mice, may further refine the pathogenesis of this phenotype.

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Table 1. Frequency and severity of testicular arteriolosclerosis by age and strain. Number in parentheses after group is total number of male mice in that group. Mean arteriolosclerosis score is the average disease severity score of all mice (including normal, score 0), for each strain.

Number in parentheses after mean arteriolosclerosis severity score is average score in affected mice only.

Figures 1-4. Arteriolosclerosis, testes, mice. Figure 1. Hyaline material expands the media of multiple medium and small intertubular arteries in a 20 month old SM/J mouse. Unaffected arteries are present, demonstrating the segmental nature of the lesions. Hematoxylin & eosin (HE). Figure 2. Abundant hyaline eosinophilic material expands the media and compresses the lumen of a small intertubular artery of a 20 month old SM/J. HE. Figure 3. Arteriolosclerosis of the arterioles in a 20 month old WSB/EiJ. HE. Figure 4. Chronic lesion with florid adventitial fibrosis in a 20 month old SM/J mouse. HE.

Figures 5-8. Arteriolosclerosis, testes, mice. Figure 5. Periodic acid Schiff (PAS) positive hyaline material expands the arterial media in a 20 month old SM/J mouse. PAS. Figure 6. Hyperplastic smooth muscle in the media of a small testicular artery of a 20 month old SM/J mouse. PAS. Figure 7. Abundant medial fibrinoid material (bright red) admixes with fibrillar collagen ~~and reticulin~~ (~~yellow~~blue) in a ~~26~~ 12 month old ~~(longitudinal)~~ SM/J mouse. ~~The internal elastic lamina (purple) is fragmented~~. ~~Movat's pentachrome~~Masson's trichome. Figure 8. Different arterial segment from the same mouse showing mature diffuse medial and adventitial fibrosis and demonstrating the variable nature of lesions within adjacent vessels. ~~Movat's pentachrome~~Masson's trichrome.

Figure 9. Testicular arteriolosclerosis by mouse strain. Adapted from Petkov *et al.* Used with permission. The phenotype of testicular HAS is present with varying frequency in multiple genetically divergent and distinct inbred mouse strains. Red boxes delineate strains with high frequency; blue boxes for strains with low frequency, and grey boxes for strains negative for the phenotype. The PWD/PhJ strain (negative, not shown) is in group 7. The RF strain in Group 1 (turquoise) was previously described to develop these lesions, but no frequency was reported.