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Systemic AA amyloidosis in island foxes (*Urocyon littoralis*): Severity and risk factors

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Abstract

Systemic amyloid A (AA) amyloidosis is highly prevalent (34%) in endangered island foxes (*Urocyon littoralis*) and poses a risk to species recovery. Although elevated serum amyloid A from prolonged or recurrent inflammation predisposes to AA amyloidosis, additional risk factors are poorly understood. Here we define the severity of glomerular and medullary renal amyloid and identify risk factors for AA amyloidosis in 321 island foxes necropsied from 1987 through 2010. In affected kidneys, amyloid more commonly accumulated in the medullary interstitium than in the glomeruli [98% (78/80) versus 56% (45/80), respectively, $p < 0.0001$], and medullary deposition was more commonly severe [19% (20/105)] as compared to glomeruli [7% (7/105), $p = 0.01$]. Univariate odds ratios (ORs) of severe renal AA amyloidosis were greater for short- and long-term captive foxes compared to free-ranging (OR=3.2, 3.7, respectively, overall $p = 0.05$) and females compared to males (OR = 2.9, $p = 0.05$). Multivariable logistic regression revealed independent risk factors for amyloid development were increasing age class (OR = 3.8, $p < 0.0001$), San Clemente Island subspecies compared to San Nicolas Island subspecies (OR = 5.3, $p = 0.0003$), captivity (OR = 5.1, $p = 0.0001$), and nephritis (OR = 2.3, $p = 0.01$). The increased risk associated with the San Clemente subspecies or captivity suggests roles for genetic as well as exogenous risk factors in the development of AA amyloidosis.

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Keywords

Amyloid A amyloidosis; island fox; logistic regression; nephritis; serum amyloid A; *Urocyon littoralis*

Endangered island foxes (*Urocyon littoralis*) have lived exclusively on the southern California Channel Islands[1,55] in genetic isolation for more than 10,000 years.[6,15,51] The foxes evolved into six island-specific subspecies with low intra-island genetic variability[15] attributed to historic population bottlenecks.[2] Recent catastrophic population declines due to non-native predators[7,40] and canine distemper[47] left some islands with as few as 15 individuals. Disease surveillance through post-mortem examination revealed a high prevalence of systemic amyloid A (AA) amyloidosis,[11] which poses a threat to species recovery.

AA amyloidosis is the most common systemic amyloidosis in animals and the second most common in humans, and develops following prolonged or cyclic elevation in the acute phase protein, serum amyloid A (SAA).[3,24,43] Transcription of SAA is up-regulated in hepatocytes in response to pro-inflammatory cytokines IL-1, IL-6 and TNF-α.[49] SAA circulates bound to high density lipoprotein (HDL)[3] and serum levels can increase up to 1000-fold in response to infection, inflammation, or tissue damage.[27] Elevated SAA concentration,[42] SAA polymorphisms or mutations,[4,14,30,57] and proteolytic cleavage of SAA into amyloidogenic fragments[53] have been proposed as mechanisms promoting AA amyloid formation.

AA amyloidosis in humans has been associated with chronic infectious diseases, such as tuberculosis and leprosy.[35] Similarly, AA amyloidosis in captive cheetahs is associated with moderate to severe chronic lymphoplasmacytic gastritis[37] from *Helicobacter sp.* infection.[10] Island foxes also have chronic infections such as the endemic intestinal parasites *Mesocestoides*[48] and *Spirocerca* sp.,[31,36] the latter causing chronic mural intestinal granulomas in island foxes and elevated serum SAA levels in dogs.[34] Other notable chronic parasitism in island foxes is due to the *Otodectes sp.* ear mite, which is common on the three southern islands and is associated with chronic otitis externa.[31]

Genetic risk factors for amyloidosis have been identified in humans and animals. In humans with familial Mediterranean fever (FMF), an autosomal recessive disease, 60 missense mutations in the pyrin gene are associated with AA amyloidosis.[48] Humans with rheumatoid arthritis have several risk factors for AA amyloidosis, including SAA1.1 (52A) and SAA1.3 (57A)[14,57] genotypes, and a single nucleotide polymorphism (SNP) in the SAA1 promoter flanking region that increases SAA transcription.[29] Similarly, in captive cheetahs, a SNP in the SAA promoter increases SAA transcription under pro-inflammatory conditions in vitro.[59]

Familial AA amyloidosis occurs in Shar Pei dogs,[9] and Abyssinian and Siamese cats,[19,33,50] but by unknown mechanisms. Highly prevalent AA amyloidosis in inbred captive black-footed cats may be a heritable trait,[46] and concurrent disease and a genetic predisposition are

proposed to influence AA amyloidosis development in the captive black-footed ferret population.[12]

AA amyloid in island foxes has a widespread tissue distribution, being present in nearly every organ except brain, most commonly kidney, spleen and oral cavity. Fox AA amyloid is congophilic, birefringent under polarized light, immunoreactive against anti-canine AA antibody, and was identified in high abundance by mass spectrometry in amyloid affected tissues.[11] Given the high prevalence of AA amyloidosis in genetically and geographically isolated island foxes, here we describe the severity of AA amyloid deposits and assess risk factors for disease.

Materials and methods

Case definition and histological analysis

The study population included free-ranging and captive island foxes (n = 321) that died between 1987 and 2010 and were necropsied as part of a disease surveillance and population monitoring program. Foxes represented all six island subspecies [Santa Cruz (*Urocyon littoralis santacruzae*), Santa Rosa (*U. l. santarosea*), San Miguel (*U. l. littoralis*), San Clemente (*U. l. clementae*), Santa Catalina (*U. l. catalinae*) and San Nicolas (*U. l. dickeyi*)]. Only carcasses with the majority of organs present and without significant autolysis were included; neonates were excluded. Animal history, gross necropsy reports, and histopathology slides were reviewed by a single board-certified veterinary pathologist (PMG) to maintain consistency in the evaluation and severity scoring. Presence of amyloid and all concurrent lesions were determined by examination of formalin-fixed, paraffin-embedded tissue sections stained with hematoxylin and eosin (HE) and Congo red to confirm amyloid by green birefringence under polarized light. A case of amyloidosis was defined as a fox with confirmed amyloid in any organ. A semi-quantitative grading scale adapted from Terio et al.[46] was used to assess severity of amyloid deposition in kidney and spleen as absent (0%), mild (<25%), moderate (25–50%), or severe (>50%) (Supplementary Table 1).

Immunohistochemistry

Immunohistochemistry for SAA was performed using the avidin-biotin-peroxidase method. Endogenous peroxidase was quenched with 3% hydrogen peroxide. Tissues were then blocked, sequentially incubated in polyclonal rabbit anti-canine AA antibody,[54,18] biotinylated goat anti-rabbit secondary, and streptavidin horse-radish peroxidase, and then visualized using a 3,3′-diaminobenzidine substrate. Positive controls included a Shar Pei dog kidney with amyloid and an island fox kidney with AA amyloid confirmed by mass spectrometry.[11] Additional controls included (i) rabbit IgG isotype substitution of the primary antibody on island fox tissues containing amyloid and (ii) primary antibody on island fox tissues lacking congophilic deposits.

Electron microscopy – in situ

Island fox spleen with severe nodular AA amyloid (confirmed by Congo red stain and immunohistochemistry) was fixed in Karnofsky’s solution, post-fixed in osmium tetroxide,

embedded in Epon resin, sectioned at 60 nm onto nickel grids, and negatively stained with saturated uranyl acetate in 50% ethanol and bismuth sub-nitrate solution. Grids were analyzed with a Zeiss EM10 Transmission Electron Microscope (TEM).

Amyloid fibril extraction and ultrastructure

AA positive spleen tissue from an island fox with severe nodular amyloidosis was stored at −80 °C and thawed immediately prior to use. A 55 mg (wet weight) piece was severed and diced with a scalpel followed by two rounds of homogenization in 20 mM Tris, 140 mM NaCl, 10 mM ethylenediaminetetraacetic acid, and 0.1% (w/v) NaN_3, pH 8.0, using a kontes pellet pestle. The sample was centrifuged at 3,000 rpm and 4 °C for 5 min, and the supernatant was carefully removed and discarded. The pellet was homogenized repeatedly with a kontes pellet pestle in pure water, on ice.[38] At the end of each homogenization step, the sample was centrifuged and the fibril-containing supernatant was carefully removed and analyzed by TEM.

A 5 μl aliquot of the fibril extract was placed onto a carbon coated formvar 200-mesh copper grid and incubated for 5 min at room temperature. The excess solution was removed by blotting the grid on Whatmann filter paper, and the grids were washed three times with 50 μl water droplets and stained three times with 50 μl of 2% (w/v) uranyl acetate solution. The grid specimens were air-dried and examined in a Jeol 1400 plus TEM operated at an acceleration voltage of 120 kV.

Risk factors

Evaluated risk factors for amyloidosis are listed in Table 2. Stratifications and grouping of data are as follows: age classes were categorized as young of the year/juvenile, young adult, adult, and geriatric based on known age or tooth eruption and wear pattern of the first upper molar[56] and age class was evaluated both as a categorical and continuous variable.

Island subspecies were divided into four groups: San Nicolas, Santa Catalina, San Clemente, and Northern islands (Santa Cruz, Santa Rosa, San Miguel). The Northern island subspecies were grouped due to geographic proximity, shared management practices, and small sample size. Four managing entities were included to assess differing population monitoring practices affecting carcass recovery and necropsy submission; these entities included the Catalina Island Conservancy (CIC), the United States Navy (Navy), the National Park Service (NPS) and North American mainland zoos (Zoo).

Housing type was categorized as free-ranging or captive. Free–ranging foxes had never been in a captive environment. The captive category was divided into short-term and long-term captivity, where “short-term” included foxes held in on-island hospitals or holding facilities for 30 days or less, and “long-term” included foxes housed in on-island breeding pens or North American mainland zoos for as long as 9 years. Days in captivity were also determined for foxes within the captive strata.

The variable “chronic inflammation” was defined by the diagnosis of one or more of the individually assessed inflammatory diseases as chronic, based on histology at the time of

death. Inflammatory diseases included otitis externa, nephritis, spirocerca granuloma, abscess/cellulitis, pneumonia, septicemia, arthritis, hepatitis, endocarditis, and enteritis.

Proportions of inflammatory diseases were calculated with the denominator reflecting the availability of the organ for examination. Neoplasia included any type of neoplasm present at the time of death. Bilateral adrenal cortical hyperplasia was used as an indicator of chronic stress and was diagnosed microscopically based on a greater than 2:1 ratio of cortex to medulla width.

Statistical analysis

Prevalence for AA amyloidosis was calculated for the individual island subspecies; differences between prevalence estimates were determined using a 2-sample z-statistic with $p \leq 0.05$ considered significant. Differences between proportions of mild, moderate and severely affected kidney and spleen were also determined using a 2-sample z-statistic with $p \leq 0.05$ considered significant.

Demographic and management factors and concurrent diseases were evaluated for their association with amyloidosis with univariate and multivariable logistic regression using statistical software (SAS, version 9.3, SAS Institute Inc., Cary NC). Univariate analyses were used to screen for risk factors to include in the multivariable model and estimate unadjusted odds ratios (ORs) and 95% confidence intervals (CIs). Factors with $p \leq 0.25$ were considered in the multivariable model[16] which was assembled with stepwise logistic regression and using methods of model fitting and assessment of interactions, as described.[16] Residuals and Hosmer-Lemeshow Goodness-of-fit-test were used to assess model fit. The final model was selected based on statistical assessments and biological plausibility. The model included adjusted ORs and 95% CIs for cases relative to non-cases as a function of the evaluated risk factors with $p \leq 0.05$ considered significant.

The association between the number of days in captivity [natural log (ln) transformation] and amyloidosis was examined as a continuous variable in the subset of captive foxes. To assess the association between amyloidosis and presence of certain diseases, some data analyses were limited to subsets of island subspecies where the diseases are present. Otitis secondary to *Otodectes sp.* ear mite infection was examined only in the southern island species where this ear mite occurs (Santa Catalina, San Clemente, San Nicolas). Associations with otitis and ceruminous gland neoplasia were examined in the Santa Catalina subspecies in which otitis is the most severe and ceruminous neoplasms were exclusively found.

The amyloid-containing kidney was chosen to assess the association between risk factors and amyloid severity because kidney is the most commonly affected organ resulting in clinical disease and mortality. The highest grade of renal amyloid, glomerular or medullary, was used in the analysis. Due to small sample size, the association between risk factors and severe amyloid deposition in kidney was examined with exact univariate logistic regression.

Results

Gross lesions and ultrastructure of AA amyloid

We characterized the macroscopic lesions in 109 cases of amyloidosis in necropsied island foxes. The kidney, oral cavity and spleen most commonly had gross lesions. In severely affected cases, the kidneys appeared diffusely pale and waxy (Figure 1) with subcapsular stippling and white medullary streaks on the cut surface. In the oral cavity, there was macroglossia (up to 2 times normal size), often with mucosal hyperplasia of glossal papillae and submucosal amyloid nodules up to 8 mm. Within the epiglottis, there were 1–3 mm nodules of amyloid that distorted the normal architecture (Figure 2). The spleen was diffusely enlarged (2 to 4 times normal size) or contained macroscopically visible white nodules of amyloid (up to 7 mm) (Figure 3).

Since nodular splenic AA amyloid is unusual, amyloid fibrils were examined ultrastructurally by TEM, both in situ and in splenic extracts. Severe nodular splenic amyloidosis (Figure 4a) was confirmed as AA by immunoreactivity to anti-canine AA antibody (Figure 4b). In situ, nodules of splenic amyloid were composed of densely packed mats of haphazardly arranged non-branching fibrils of approximately 10 nm diameter (Figure 5). The extracted fibrils also possessed a linear and non-branching structure, however they lacked high regularity and did not exhibit a uniform fibril width when measured at different positions along the same fibril (Figure 6). Instead, fibrils often appeared broken or splintered and possessed multiple aligned protofilaments. Consequently, the fibril width differed considerably from 10 to almost 50 nm. Only some fibrils exhibited a well-resolved crossover structure (constriction at the site of fibril twists) (Figure 6).

Renal and splenic amyloid severity—In cases with renal amyloidosis (n = 80), medullary amyloid was more common than glomerular amyloid [98% (78/80)] and 56% (45/80), respectively, $p < 0.0001$]. Because renal amyloid can lead to kidney failure, we characterized the severity of renal AA amyloid in all amyloidosis cases with available kidney (n = 105). The degree of amyloid deposition in glomeruli and medulla was designated as absent, mild, moderate, or severe, reflecting 0%, <25%, 25–50%, or >50% amyloid deposition (Supplementary Table 1). Amyloid was absent from glomeruli and medulla in 57% and 26% of kidneys, respectively. Glomerular amyloid deposits were most often mild (30%); only 7% of amyloid cases had severe deposition. Similarly, medullary amyloid deposits were most often mild (43%), and were less frequently moderate (12%) or severe (19%) (Figures 7 – 16).

In amyloid cases with kidney available for examination, renal amyloidosis caused or contributed to death in 18% of foxes (19/105). This may under-represent the mortality due to renal amyloidosis as many of these amyloid cases (n = 39) died prematurely from vehicular trauma. Among non-trauma related deaths, 29% (19/66) of amyloid cases died from renal amyloidosis.

In the spleen (n = 98), the proportions of mild (19%), moderate (19%) and severe (18%) amyloid deposition were nearly equal, while the remaining (43%) cases had no splenic amyloid (Figure 13 – 16).

Prevalence and risk factors for AA amyloidosis

Prevalence of AA amyloidosis in island fox subspecies—To estimate prevalence, we examined the necropsied population of island foxes (n=321) that died between 1987 and 2010 and were juveniles or older. The overall prevalence of amyloidosis in this population was previously estimated at 34% (109/321).[11] Prevalence among each island subspecies was further evaluated in the present study and ranged from approximately 23 – 50% [San Nicolas (22.6%), Santa Catalina (29.5%), Santa Cruz (33.3%), Santa Rosa (36.4%), San Clemente (38.9%), and San Miguel (50.0%)] (Table 1). The prevalence of amyloidosis in the San Clemente subspecies was significantly higher than in the San Nicolas subspecies ($p = 0.02$). Prevalence estimates were not significantly different between the other island subspecies or between the southern or northern island groups.

Most foxes examined were free-ranging (n = 253); fewer were housed for any time in captivity (n = 66). Prevalence of AA amyloidosis among foxes housed in captivity was significantly higher compared to the free-ranging population [59% (39/66) vs. 27% (69/253), respectively, $p < 0.0001$].

Concurrent inflammatory diseases—Co-morbid inflammatory lesions at the time of death included chronic otitis externa associated with *Otodectes sp.* ear mite infection, which was present in the majority of cases (88.8%) from the southern islands. Nephritis, including glomerulonephritis, pyelonephritis, interstitial nephritis and pyelitis, was the next most common group of lesions (39.6%), followed by mural intestinal or mesenteric granulomas due to *Spirocerca sp.* infection (24.0%), and trauma-associated abscessation and cellulitis (20.2%). Less common concurrent inflammatory lesions were pneumonia (14.6%), sepsis (5.6%), arthritis (5.5%), hepatitis (5.0%), endocarditis (3.9%), and enteritis (3.9%). Animals were considered to have chronic inflammation if they had histologic evidence of chronic, active inflammation at the time of death, which was 83.5% of the amyloid cases and 90.6% of non-cases (Table 2).

Risk factors for AA amyloidosis—To identify risk factors for amyloidosis in the island foxes, 18 variables were assessed for their association with the presence of AA amyloid (Table 2). Eleven variables met the criteria for consideration in the multivariable analysis ($p \leq 0.25$) including age class, island subspecies, island management type, housing type, neoplasia, adrenal cortical hyperplasia, chronic inflammation, and specific inflammatory diseases including otitis, nephritis, abscesses/cellulitis, arthritis, and enteritis. Based on associations in the univariate analysis and to improve statistical efficiency, the final model included age class as a continuous predictor and short-term and long-term captivity were combined into a single category, denoted captive. The final adjusted model included four independently significant variables: age class, island subspecies, captivity, and nephritis (Table 3). There was no evidence of lack-of-fit (Hosmer-Lemeshow Goodness-of-fit $p = 0.47$), or identified confounders or interactions.

Our regression model showed that successively increasing age classes were associated with amyloidosis ($p < 0.0001$). The odds of disease increased 3.8-fold for each increasing age class. Adjusting for age and other variables in the model, the San Clemente Island fox

subspecies had 5.3 times higher odds of having amyloidosis (p = 0.0003) when compared to the San Nicolas subspecies. Foxes housed in captivity were 5.1 times more likely to have amyloidosis than free-ranging foxes (p = 0.0001); however, the risk did not increase with longer time in captivity (p = 0.50) in an adjusted analyses among the captive subset. The presence of any nephritis (pyelonephritis, interstitial nephritis, glomerulonephritis) was significantly associated with amyloidosis (OR = 2.3, p = 0.01). Whether nephritis preceded or was a sequela of amyloidosis is not known. Because previous work has shown nephritis occurs in dogs with renal amyloidosis[41] and cheetahs with chronic renal disease have elevated circulating SAA,[8] we examined the relationship between nephritis and renal amyloidosis and found no association in island foxes (p = 0.3).

An association between neoplasia and AA amyloidosis was initially identified in the univariate analysis, but was explained by the increased occurrence of neoplasia with increasing age and was no longer significant in the adjusted model.

Risk factors for severe renal AA amyloidosis—Among the cases of renal amyloidosis (n = 80), the same risk factors were assessed for an association with severe renal amyloidosis by exact univariate logistic regression and revealed that female foxes and foxes in short- and long-term captivity had significantly higher odds of severe renal amyloid when compared to males and free-ranging foxes (OR: 2.9, 3.2, and 3.7, respectively, all p-values < 0.05) (Supplementary Table 2). Management type was also significantly associated with severe renal amyloidosis (p = 0.03) when comparing zoo foxes to those managed by CIC. Small sample size did not allow for further assessment of this association; however severe renal amyloid in zoo foxes may be due to longer life in captivity with clinical care. No other variables had a significant association with severe renal amyloid. Adjusted, multivariable analyses were not performed due to the small sample size and lack of statistical power.

Island-specific risk factors for AA amyloidosis

Otodectes sp.- associated chronic otitis externa occurred only in the Southern island foxes (Santa Catalina, San Clemente, San Nicolas) but was not associated with amyloidosis (p = 0.3). Ceruminous gland tumors were only identified in Santa Catalina foxes and were not significantly associated with amyloidosis (p = 0.3).

Discussion

The island fox is an endangered species with a high prevalence of AA amyloidosis. Here we define the degree of amyloid deposition in the most commonly affected organs, kidney and spleen, and identify the risk factors for disease. Independent risk factors for AA amyloidosis were age class, San Clemente Island subspecies, captivity, and nephritis.

In island foxes, the kidney most commonly contains AA amyloid,[11] similar to humans and many animal species with systemic AA amyloidosis.[20,28,37,39,41,46] Here we found that both glomerular and medullary amyloid deposits were common in affected fox kidneys, however medullary deposits were nearly universal, similar to Shar Pei dogs,[9,41] Abyssinian cats,[19,33] and captive cheetahs[37] and black-footed cats.[46]

The severity of renal amyloid deposition in island foxes was typically mild. In contrast, renal AA amyloidosis in captive cheetahs, black-footed cats, and black-footed ferrets, which also have a high prevalence of systemic AA amyloidosis, is typically severe.[12,37,46] This difference is most likely attributable to most island foxes being free-ranging and subject to loss at younger ages from predation and vehicular trauma and lack of medical treatment. Captive island foxes were more likely to have severe renal amyloidosis.

Prolonged or cyclic elevation of circulating SAA typically precedes the deposition of AA amyloid. Chronic elevations in SAA are secondary to infectious, immune-mediated, neoplastic and familial disease. Most island foxes died with evidence of a chronic and ongoing inflammatory disease, *Otodectes sp.* or *Spirocerca sp.* infection, or a tumor. Other than nephritis, there was no significant association between an acute or chronic inflammatory disease and amyloidosis when controlling for other demographic or management factors. This finding was not surprising given that 90.6% of amyloid-negative foxes also had evidence of chronic inflammation. While inflammation likely predisposes to AA amyloidosis and nephritis was significantly associated with disease, additional endogenous or environmental co-factors may also contribute to the high prevalence of AA amyloidosis in island foxes.

A genetic or exogenous factor may promote the development of AA amyloidosis in island foxes, particularly considering the 5.3-fold increased odds of amyloidosis for the San Clemente versus San Nicolas subspecies and the absence of reported amyloidosis in mainland gray foxes, the island fox's closest relative.[21] Possible genetic associations with amyloidosis could be due to 1) a mutation in the SAA gene(s) resulting in a highly amyloidogenic SAA sequence, or 2) a mutation in the promoter, enhancing elements, or transcription factors resulting in elevated or prolonged SAA transcription. The amyloidogenic SAA1.1 isoform in humans and island fox SAA both have a high predicted aggregation potential at amino acid segment 2–7.[11,25] An SAA promoter mutation is also a possibility, as described in the cheetah.[59]

Captivity was a significant risk factor for AA amyloidosis in island foxes, independent of age and subspecies; however, increased days in captivity was not associated with any increase in risk. This suggests some aspect of the initial exposure to the captive environment may account for the increased risk for disease. One possible link between exposure to a captive environment and AA amyloidosis is the potential for transmission of AA amyloid by a prion-like mechanism through a seeding-nucleation process.[5,32] A captive environment contaminated with AA amyloid fibrils could expose an animal to an AA amyloid seed. In mice injected with a pro-inflammatory stimulus, such as silver nitrate, AA amyloid deposition is accelerated by inoculation of amyloid fibrils (amyloid enhancing factor) isolated from amyloidotic organs[23,26] or from cheetah feces.[58] The potential for island fox AA amyloid to transmit horizontally from animal to animal should be considered.

Another possible explanation for captivity as a risk factor is the stress-induced production of glucocorticoids, which are known to enhance IL-1- and IL-6-induced SAA transcription.[17,44,49] Elevated fecal cortisol levels have been shown in captive compared to wild cheetahs and cheetahs on- versus off-exhibit.[45,52] Additionally, the social stress of

crowded housing is associated with AA amyloidosis in Syrian hamsters and mice in the laboratory setting.[13,22] Thus, captive foxes may experience enhanced SAA transcription from stress-induced glucocorticoid production that could promote amyloidosis.

The risk factors for amyloidosis suggest genetic and exogenous factors promote disease, however, the underlying mechanisms remain unclear. Further investigation of the molecular pathogenesis of amyloidosis is warranted to determine the role of genetic mutations, epigenetic changes, and environmental influences on AA amyloidosis development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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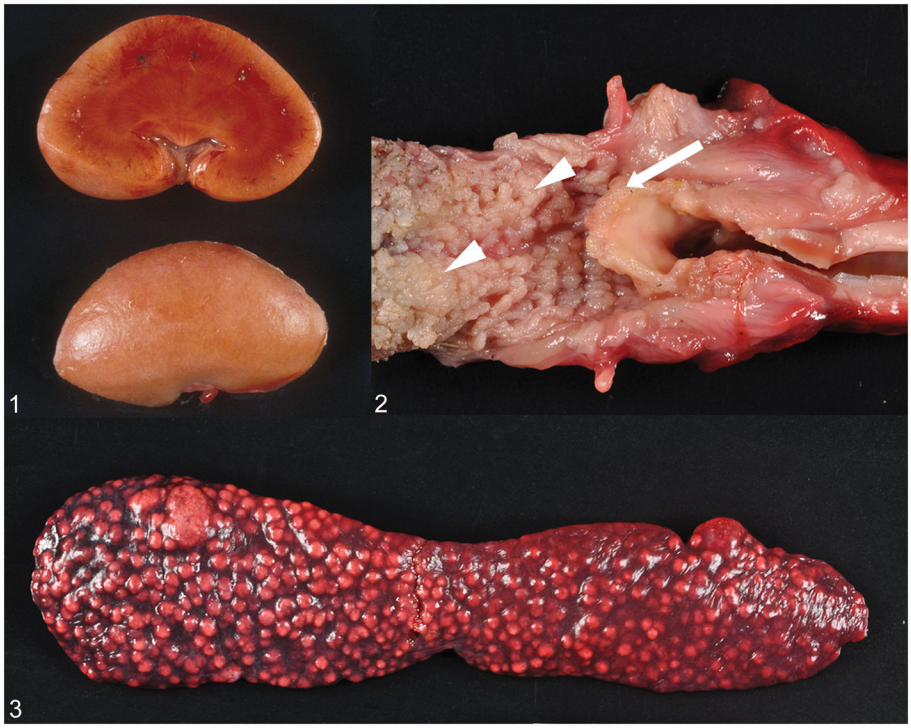
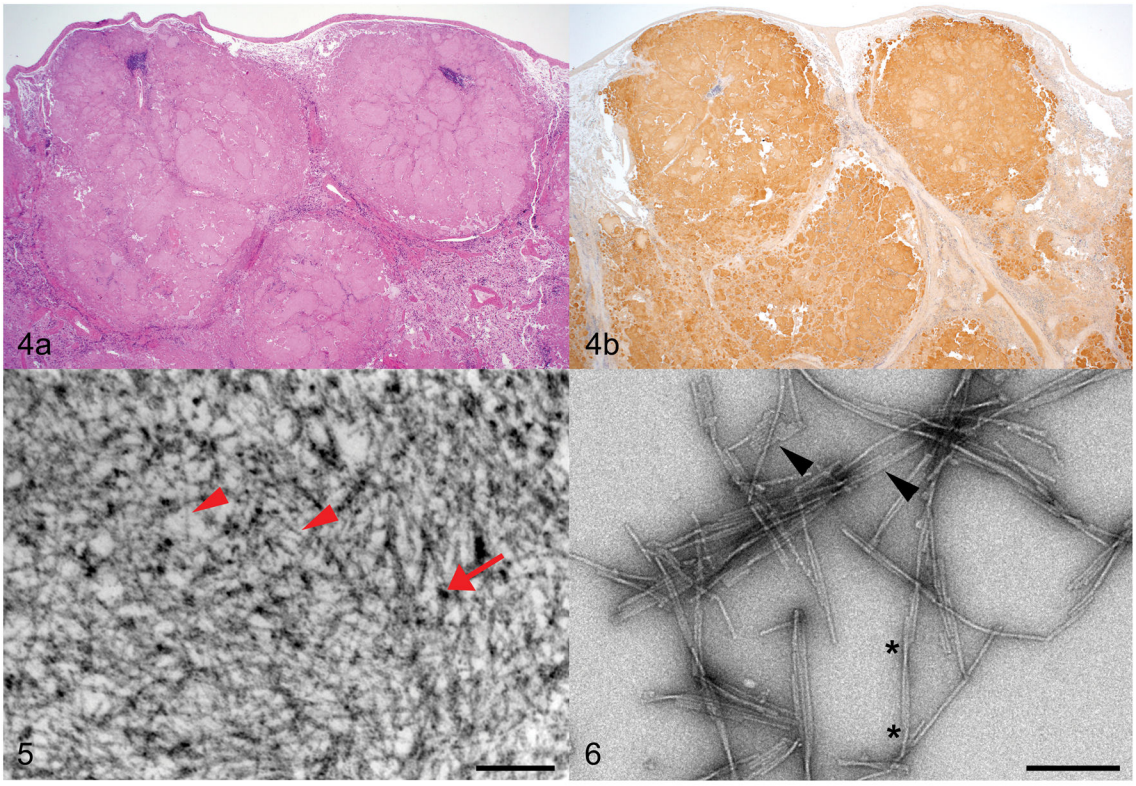


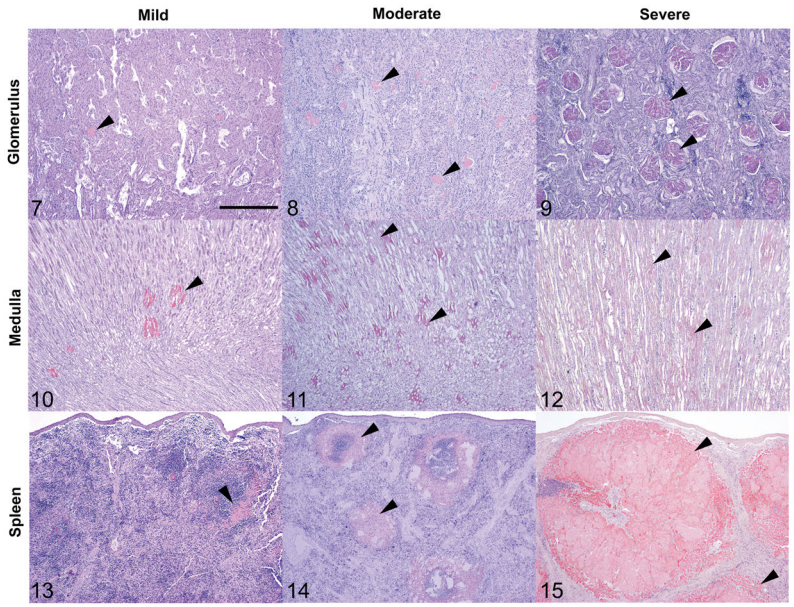
Figures 1 – 3.

Appearance of AA amyloidosis in island fox organs. Figure 1: The kidneys were diffusely infiltrated by amyloid and appeared pale tan, dry, and waxy. Figure 2: The tongue and epiglottis (arrow) were markedly thickened and distorted by raised nodules composed of amyloid and hyperplastic, sometimes papillary, mucosa (arrowheads). Figure 3: Large nodules of amyloid enlarge and replace splenic parenchyma.



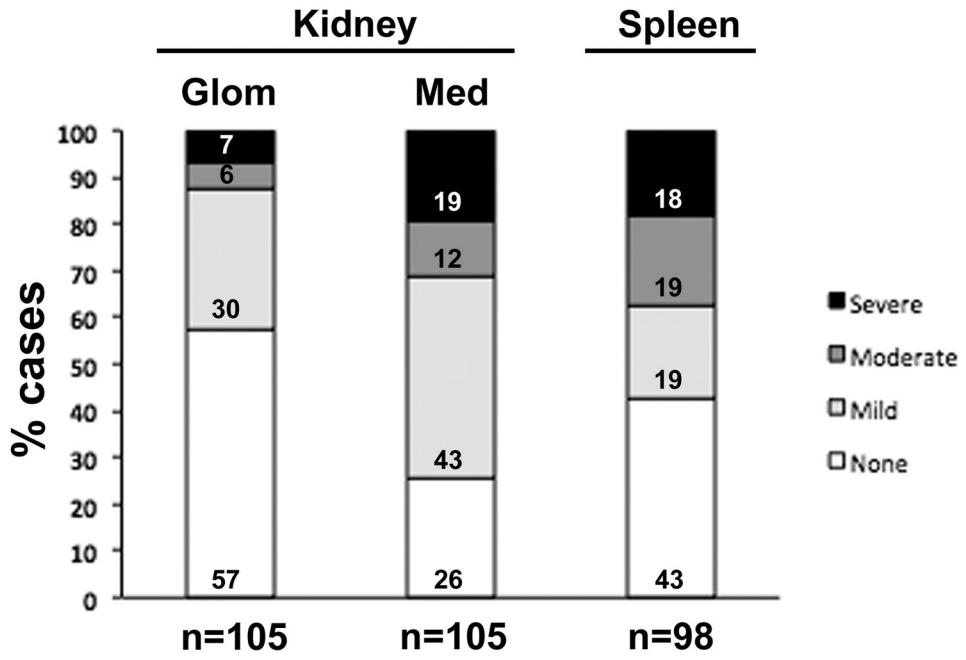
Figures 4 – 6.

Microscopic and ultrastructural appearance of severe nodular amyloidosis in the spleen of island fox. Figure 4a: Large nodules of amyloid replace and distort the majority of splenic architecture. Hematoxylin and eosin. Figure 4b: Nodules of splenic amyloid are immunoreactive to anti-canine AA antibody. **Figure 5:** AA amyloid nodule in the spleen shows the architecture is replaced by a meshwork of haphazardly arranged, approximately 10 nm diameter, unbranching fibrils (red arrowheads), occasionally in cross-section (red arrow). Scale bar = 150 nm. Transmission electron microscopy (TEM), uranyl acetate. **Figure 6:** Splenic AA amyloid extracts reveal linear, non-branching fibrils from 10 – 50 nm diameter, sometimes with multiple aligned protofilaments (black arrowheads) and occasional well-resolved crossover structure (black asterisks). Scale bar = 250 nm. TEM, uranyl acetate.



Figures 7 – 15.

Mild, moderate, and severe AA amyloidosis (black arrowheads), kidney and spleen, island fox. Congo red. **Figure 7 – 9.** Kidney, cortex. Mild (**7**), moderate (**8**), or severe (**9**) amyloid aggregates are within or replace glomeruli. **Figure 10 – 12.** Kidney, medulla. Mild (**10**), moderate (**11**), or severe (**12**) amyloid aggregates expand the medullary interstitium. **Figure 13 – 15.** Spleen. There is mild (**13**) amyloid deposition surrounding lymphoid follicles and within vessel walls. Moderate (**14**) amounts of amyloid encircle lymphoid follicles. Severe (**15**) nodular amyloidosis replaces 90% of splenic parenchyma.



16

Figure 16.

Proportions of cases with varying degrees of AA amyloid deposition in glomeruli, renal medulla and spleen. When present, renal amyloid was most commonly mild for both the glomeruli and medulla. Mild, moderate and severe amyloidosis occurred in nearly equal proportions in the spleen. Proportions were calculated out of total organs available for examination in cases of amyloidosis.

Table 1

Estimated prevalence of AA amyloidosis in necropsied island foxes (n=321).

Island location	Island fox subspecies	No. foxes with amyloidosis	No. foxes in necropsy population	% amyloidosis	(95% CI)[a]
South	Santa Catalina	18	61	29.5	(19.1 – 41.8)
	San Clemente	65	167	38.9[b]	(31.8 – 46.5)
	San Nicolas	14	62	22.6[b]	(13.5 – 34.2)
	All Southern subspecies	*97*	*290*	*33.4*	(*28.2 – 39.0*)
North	Santa Cruz	4	12	33.3	(11.6 – 62.3)
	Santa Rosa	4	11	36.4	(12.8 – 66.4)
	San Miguel	4	8	50.0	(18.4 – 81.6)
	All Northern subspecies	*12*	*31*	*38.7*	(*22.9 – 56.5*)
Total		**109**	**321**	**34.0**	**(28.9 –39.3)**

Abbreviations: CI, confidence interval.

[a] Mid-P exact

[b] Significantly different prevalence esimates (p=0.02). There were no other significant prevalence differences.

Table 2

Univariate logistic regression analyses of the relationship between AA amyloidosis in island foxes and evaluated variables (n=321).

		Cases (n=109)		Non-cases (n=212)				
Variable	**Strata**	**No.**	**%[a]**	**No.**	**%[a]**	**OR**	**(95% CI)**	**P-value[b]**
Sex	Female	57	52.3	100	47.4	1.2	(0.8 – 1.9)	0.4062
	Male	52	47.7	111	52.6	Reference		
	Unknown	0		1				
Age class[c]	Geriatric	47	43.5	27	13.0	51.6	(14.9 – 179.2)	<.0001[d]
	Adult	39	36.1	41	19.8	28.2	(8.2 – 96.7)	
	Young adult	19	17.6	50	24.2	11.3	(3.2 – 40.0)	
	Juvenile	3	2.8	89	43.0	Reference		
	Unknown	1		5				
Island subspecies	San Clemente	65	59.6	102	48.1	2.2	(1.1 – 4.3)	0.1051[d]
	Santa Catalina	18	16.5	43	20.3	1.4	(0.6 – 3.2)	
	Northern islands	12	11.0	19	9.0	2.2	(0.8 – 5.5)	
	San Nicolas	14	12.8	48	22.6	Reference		
Management type[e]	CIC	18	16.5	1	0.5	0.1	(0.01 – 0.6)	0.1064[d]
	Navy	73	67.0	149	70.3	0.1	(0.01 – 0.7)	
	NPS	12	11.0	19	9.0	0.1	(0.01 – 1.0)	
	Zoo	6	5.5	43	20.3	Reference		
Housing type[f]	Short-term captivity	17	15.9	8	3.8	5.7	(2.3 – 13.7)	<0.0001[d]
	Long-term captivity	22	20.6	19	9.0	3.1	(1.6 – 6.1)	
	Free-ranging	69	64.5	184	87.2	Reference		
	Unknown	1		1				
Neoplasia	Yes	18	16.5	6	2.8	6.7	(2.7 – 19.1)	<0.0001[d]
	No	91	83.5	206	97.2	Reference		
Adrenal cortical hyperplasia	Yes	9	9.0	8	4.0	2.4	(0.9 – 6.6)	0.09337[d]
	No	91	91.0	192	96.0	Reference		
	Unknown	9		12				
Chronic inflammation	Yes	91	83.5	192	90.6	0.5	(0.3 – 1.1)	0.0711[d]

		Cases (n=109)		Non-cases (n=212)				
Variable	**Strata**	**No.**	**%[a]**	**No.**	**%[a]**	**OR**	**(95% CI)**	**P-value[b]**
Otitis externa[g]	No	18	16.5	20	9.4	Reference		
	Yes	71	88.8	173	92.0	0.7	(0.3 – 1.6)	0.3929
	No	9	11.3	15	8.0	Reference		
	Unknown	17		5				
Nephritis	Yes	40	39.6	57	27.3	1.7	(1.1 – 2.9)	0.0307[d]
	No	61	60.4	152	72.7	Reference		
	Unknown	8		3				
Spirocerca granuloma	Yes	25	24.0	58	27.6	0.8	(0.5 – 1.4)	0.5049
	No	79	76.0	152	72.4	Reference		
	Unknown	5		2				
Abscess or cellulitis	Yes	22	20.2	18	8.5	2.7	(1.4 – 5.4)	0.0038[d]
	No	87	79.8	194	91.5	Reference		
Pneumonia	Yes	15	14.6	24	11.5	1.3	(0.6 – 2.6)	0.4432
	No	88	85.4	185	88.5	Reference		
	Unknown	6		3				
Septicemia	Yes	6	5.6	10	4.7	1.2	(0.4 – 3.4)	0.7312
	No	101	94.4	201	95.3	Reference		
	Unknown	2		1				
Arthritis	Yes	6	5.5	1	0.5	12.2	(1.8 – 285.9)	0.0075[d]
	No	103	94.5	211	99.5	Reference		
Hepatitis	Yes	5	5.0	7	3.3	1.5	(0.4 – 5.0)	0.4932
	No	95	95.0	202	96.7	Reference		
	Unknown	9		3				
Endocarditis	Yes	4	3.9	10	5.0	0.8	(0.2 – 0.3)	0.6681
	No	99	96.1	191	95.0	Reference		
	Unknown	6		11				
Enteritis	Yes	4	3.9	2	1.0	4.2	(0.8 – 23.2)	0.1018[d]
	No	98	96.1	205	99.0	Reference		
	Unknown	7		5				

Abbreviations: OR, odds ratio; CI, confidence interval; Northern islands - Santa Cruz, San Miguel and Santa Rosa combined; CIC, Catalina island conservancy; NPS, National park service; Navy, United States Navy; Zoo, North American mainland zoos.

[a] Percent of known values.

[b] P-value (Wald statistic for Type 3 contrasts) for association between variable and amyloidosis.

[c] Age class was also evaluated as a continuous predictor of amyloidosis; OR = 2.9 (95% CI: 2.248 – 3.852), $p < 0.0001$.

[d] Variable considered for multivariable logistic regression model ($p < 0.25$).

[e] Management type was excluded from the multivariable model due to overlap with island subspecies in 3 of 4 categories.

[f] Included a separate analysis among the subset of animals housed in captivity with known days in captivity (n = 66); no association between days in captivity (log transformed) and amyloidosis was identified (OR = 0.933, CI = 0.762 – 1.143, p = 0.5047).

[g] Otitis externa was evaluated only in island fox subspecies at risk for *Otodectes sp.* ear mite infection, Santa Catalina, San Clemente, San Nicolas (n = 290).

Table 3

Multivariable logistic regression model for the association between AA amyloidosis in island foxes and identified risk factors (n=305)[a].

Variable	β Coefficient	SE	OR	95% CI	P-value
Age class (continuous)	1.3	0.2	3.8	(2.7 – 5.3)	<0.0001
Island Subspecies					
San Clemente	1.7	0.5	5.3	(2.2 – 13.2)	0.0003
Santa Catalina	−0.2	0.5	0.8	(0.3 – 2.3)	0.6938
Northern island	0.1	0.7	1.1	(0.3 – 4.0)	0.8879
San Nicolas	Reference				
Captivity	1.6	0.4	5.1	(2.2 – 11.5)	0.0001
Nephritis	0.8	0.3	2.3	(1.2 – 4.3)	0.0129

Abbreviations: SE, standard error; OR, odds ratio; CI, confidence interval.

[a]No evidence for lack of fit (Hosmer-Lemeshow goodness-of-fit test, p = 0.47).