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*Case Study*

# Allelic Diversity in the Serum Amyloid A2 Gene and Amyloid A Amyloidosis in a Breeding Colony of Zebra Finches (*Taeniopygia guttata*)

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**A high incidence of amyloid A (AA) amyloidosis was observed in the research breeding colony of zebra finches at our institution. Some birds with hepatic AA amyloidosis were asymptomatic for comorbid conditions frequently associated with the development of AA amyloidosis, whereas other birds with comorbid conditions failed to develop AA amyloidosis, suggesting a potential genetic component to the disease. Sequencing the *SAA2* gene from 20 birds yielded 18 distinct sequences that coded for 5 isoforms of the protein. Most of the amino acid substitutions are unlikely to affect the protein's structure or function, but 2 changes—R52L and V84M—were predicted to be disruptive. In particular, R52 is highly conserved across vertebrates, with only arginine or lysine found at this position in reported sequences to date. The atypical R52L substitution occurred in 2 otherwise healthy birds with hepatic AA amyloidosis, supporting the idea that this change is pathogenic.**

**Abbreviations:** AA, amyloid A; SAA, serum amyloid A

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Serum amyloid A (SAA) is a highly conserved apolipoprotein that participates as a major acute-phase reactant of the innate immune response in vertebrates.[32] Other functions of the SAA protein are incompletely understood, but it is believed to be involved in lipid metabolism and degradation of the extracellular matrix.[20,32] SAA circulates bound to HDL and is normally measured at 20 to 50 µg/mL in the blood of healthy adults.[28] To maintain homeostasis and restore normal physiologic function in humans and animals, the plasma concentration of SAA can increase 1000 times above baseline levels within 24 h in response to tissue damage, inflammation, infection, and trauma.[28,32] During the acute-phase response, expression of the *SAA* gene is upregulated, primarily in hepatocytes, in response to proinflammatory cytokines IL1β, IL6, TNF, and INFγ.[11,25,34] *SAA* belongs to a family of 5 or more genes that have been duplicated during evolution. Most mammals have several SAA genes, which are designated by number (*SAA1*, *SAA2*, *SAA3*, and so forth).[32] Four *SAA* genes have been identified in humans, and 5 have been identified in mice.[4,32] Zebra finches and all other birds investigated to date have a single *SAA* gene,[13,20,31] which is homologous to mammalian *SAA2*.

Sustained or chronically intermittent elevations in plasma concentrations of SAA are linked to the extracellular accumulation of amyloid A (AA) fibrils in various tissues, causing AA amyloidosis in humans and animals.[22,25,34] Precursor proteins of SAA1 and SAA2 form AA fibrils in humans, whereas only the SAA2 precursor yields fibrils in the mouse.[4] The pathogenesis of AA fibril formation is not well understood but is thought to be linked to high plasma concentrations of SAA, which overwhelm cellular quality-control mechanisms and metabolic capabilities, leading to misfolding of the SAA protein into β-pleated sheets, subsequent amyloid fibril formation and aggregation, and accumulation of amyloid deposits in the affected tissues.[3-5,19] The formation of an initial amyloid fibril is believed to hasten the subsequent fibrillation process, acting as a seed under inflammatory conditions.[21] Visceral organs, such as the liver, spleen, and kidneys are most frequently affected, but AA amyloidosis has been reported to occur in all tissues except the brain parenchyma.[3,15,25]

Chronic inflammatory illnesses, infections, various types of neoplasia, trauma, overcrowding, and stress have been associated with comorbid AA amyloidosis in humans and animals.[10,20,32,34] Our interest in the zebra finch *SAA2* gene was motivated by the high incidence of AA amyloidosis diagnosed over several years (2010 through 2015) in our institutional zebra finch research-breeding colony.[30] Many of the birds affected with AA amyloidosis had clinical conditions historically associated with the development of comorbid AA amyloidosis (pododermatitis, mycobacteriosis, chronic infections, neoplasia, and trauma). Others might have been subject to stress, which is linked to AA amyloidosis in humans and animals.[10,14,24] However, some birds diagnosed with AA amyloidosis showed minimal to no apparent comorbidity, and others with obvious clinical conditions did not develop amyloid deposits. The lack of correlation between the development of AA amyloidosis and comorbidities prompted speculation regarding possible genetic predisposition for developing or resisting AA amyloidosis caused by mutations in *SAA2*. To test this idea, we sequenced *SAA2* from 20 individual zebra finches, thus revealing 5 coding variants and abundant allelic diversity in our institutional breeding colony.

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## Materials and Methods

**Selection of cases.** ***Group 1.*** A group of 83 zebra finches was randomly selected from a larger group of approximately 150 birds that had been culled by using IACUC-approved methods from a colony of approximately 700 animals prior to its transport from University of Massachusetts Medical School to another university. The 83 selected birds were necropsied and assessed for body condition and gross lesions. The livers were removed and divided into 2 samples. One half of each liver was frozen for DNA isolation, and the other half was fixed in 10% neutral buffered formalin and embedded in paraffin for histology. Fifteen of these bird livers were randomly selected for *SAA2* sequencing and designated as group 1 birds.

***Group 2.*** This group of 5 birds had been diagnosed with systemic or hepatic AA amyloidosis and was described in a previous case report.[30] Paraffin-embedded tissues were the only materials available for these animals.

**Mycobacterial testing.** Mycobacteria species were identified by PCR analysis (Animal Genetics, Tallahassee, FL) or by using standard Ziehl–Neelsen methodology to detect acid-fast organisms.

**PCR amplification and DNA sequencing.** Liver samples were digested overnight at 50 °C in 100 mM NaCl, 1% SDS, 5 mM EDTA, 10 mM Tris (pH 8.0), and 0.2 mg/mL proteinase K. SDS was removed by addition of 1/3 volume of 4.21 M NaCl, 0.63 M KCl, 10 mM Tris (pH 8.0); samples were then incubated on ice for 10 min, followed by centrifugation. DNA was ethanol-precipitated from the supernatant and resuspended in 10 mM Tris (pH 8.0), 1 mM EDTA. DNA was isolated from paraffin-embedded tissue by using the same method after removal of paraffin by using xylene extraction.

Exons were amplified by using Go*Taq* (Promega, Madison WI) and the following primers. Exon 3 did not amplify well and required reamplification by using nested primers. The products were gel-purified (Qiagen, Hilden, Germany) and Sanger-sequenced (GeneWiz, Cambridge, MA) by using the same primers as for amplification. Primers for amplifying exon 1 were TpSAA2_exon1F (5′ TGC TTT TGT TGT GGA GCT TG 3′) and TpSAA2_exon1R (5′ GCA CCA ATG ACT GCT GGT AAG 3′); those for exon 2 were TpSAA2_exon2F (5′ TCA GCT CCT GAC TGA GGT TG 3′) and TpSAA2_exon2R (5′ CTC CCC TCT GCT GTC CTT C 3′); and those for exon 3 were TpSAA2_exon3F_2 (5′ GCA TGT GCT TTC ATC TCA CC 3′) and TpSAA2_exon3R_2 (5′ GAC CCT GAC GGG ATA GTG C 3′). Primers for product reamplification were TpSAA2_exon3F_4 (5′ TTC GAT GCA GTC TGT TCA GG 3′) and TpSAA2_exon3R_4 (5′ GCG AAG GGA ACA GTT CTC AG 3′).

**Histology and immunohistochemistry.** ***Hematoxylin–eosin and Congo red tissue staining.*** Liver tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and processed for staining with hematoxylin and eosin. Liver sections (thickness, 5 µm) were evaluated for potential amyloid deposits (extracellular eosinophilic material) by using light microscopy. Tissues identified with putative amyloid were cut into sections (thickness, 10 µm), stained with Congo red (Bennhold),[9] and further evaluated microscopically for distinctive staining properties (salmon red) of amyloid. Liver sections displaying congophilia were viewed with a polarizing filter to assess green or yellow birefringence, which is characteristic of amyloid aggregates. Samples exhibiting birefringence were evaluated by using immunohistochemistry and fluorescence microscopy to detect amyloid A protein. Histopathology was performed by board-certified veterinary and human pathologists.

***Immunofluorescence.*** Paraffin sections were dewaxed (Safe-Clear , Fisher Scientific, Hampton, NH), rehydrated with graded aqueous solutions of isopropanol, and underwent antigen retrieval in 10 mM sodium citrate (pH 6) in an autoclave (250 °F, 40 min). Sections were brought to ambient temperature and treated with blocking solution (4% nonimmune goat serum, 0.1% Triton X100, 0.05% SDS, and 0.1% fish skin gelatin [Sigma, St Louis, MO] in TBST [0.05% Tween 20 in Tris-buffered saline, pH 7.4]) for 30 min, subsequently washed with TBST, and then exposed to primary antibodies (dilution, 1:300; rabbit antiduck amyloid A, gift of Dr JT Guo, Drexel University College of Medicine, Philadelphia, PA) overnight at 4 °C. The next day, the sections were washed with TBST, incubated with Alexa Fluor-conjugated secondary antibodies (Life Technologies, Grand Island, NY) for 30 min at 22 °C, washed with TBST, and rinsed with TBS. The antibodies were brought to their working dilutions by using 0.1% fish skin gelatin in TBS. The sections were then dipped for 5 s in DAPI (1 µg/mL in TBS), rinsed with TBS, and mounted by using Prolong Gold (Life Technologies).

***Microscopy and images.*** Images of Congo red–stained tissue sections were obtained through photomicroscopy (Spot Insight QE camera, Spot Software S2, and Eclipse microscope; Nikon, Tokyo, Japan). Green birefringence was evaluated and photographed by using a polarizing filter. Immunofluorescent images were acquired by using an Orca ER camera (Hamamatsu Photonics, Hamamatsu, Japan) on an Axiovert 200M (Zeiss, Gottingen, Germany) microscope. Images were captured by using Openlab (Lexington, MA) and adjusted for contrast in Photoshop (Adobe, San Jose, CA). The photos were taken under identical conditions and manipulated equally.

**Web-based algorithms.** ***PROVEAN and PolyPhen2.*** PROVEAN[27] and PolyPhen2[26] rate the functional effects of sequence variations, by using homologous sequence retrieval and δ alignment scores[6,8] and physical and comparative considerations, respectively.[1,8]

***Clustal Omega multiple-sequence alignment.*** The NCBI zebra finch SAA2 reference protein sequence was aligned by using Clustal Omega[12] to SAA2 proteins from 35 other vertebrate species (mostly mammals and birds).

## Case Series

This case study includes 2 groups of zebra finches from a breeding colony housed at University of Massachusetts Medical School. The birds belonged to a research lab that created genetically modified animals to investigate vocal learning. The colony was deliberately managed in an outbred fashion by periodically introducing wild-type zebra finches purchased from different vendors.

Group 1 animals consisted of 15 birds (nos. 6 through 20), 8 male and 7 female, that ranged from 2 to 4 y of age. Of these 15 birds, 9 (nos. 6 through 9 and 16 through 20) were mosaic for the laboratory-generated DNA construct; the remaining 6 (nos. 10 through 15) were wildtype. Nine birds were diagnosed with hepatic AA amyloidosis due to histologic analysis showing strong Congo red staining, yellow–green birefringence under polarized light, and strong immunofluorescence staining with an antiduck amyloid A antibody (Figure 1). In these 9 affected birds, parenchymal AA deposition ranged from minimal to marked, and perivascular AA deposits ranged from minimal to moderate in 6 of the 9 affected birds. Subsequent to histologic assays and analysis, the remaining 6 birds in group 1 were considered negative for AA amyloidosis (Table 1).

Nine of the 15 birds were ostensibly healthy with a normal body condition (keel only slightly palpable, no significant

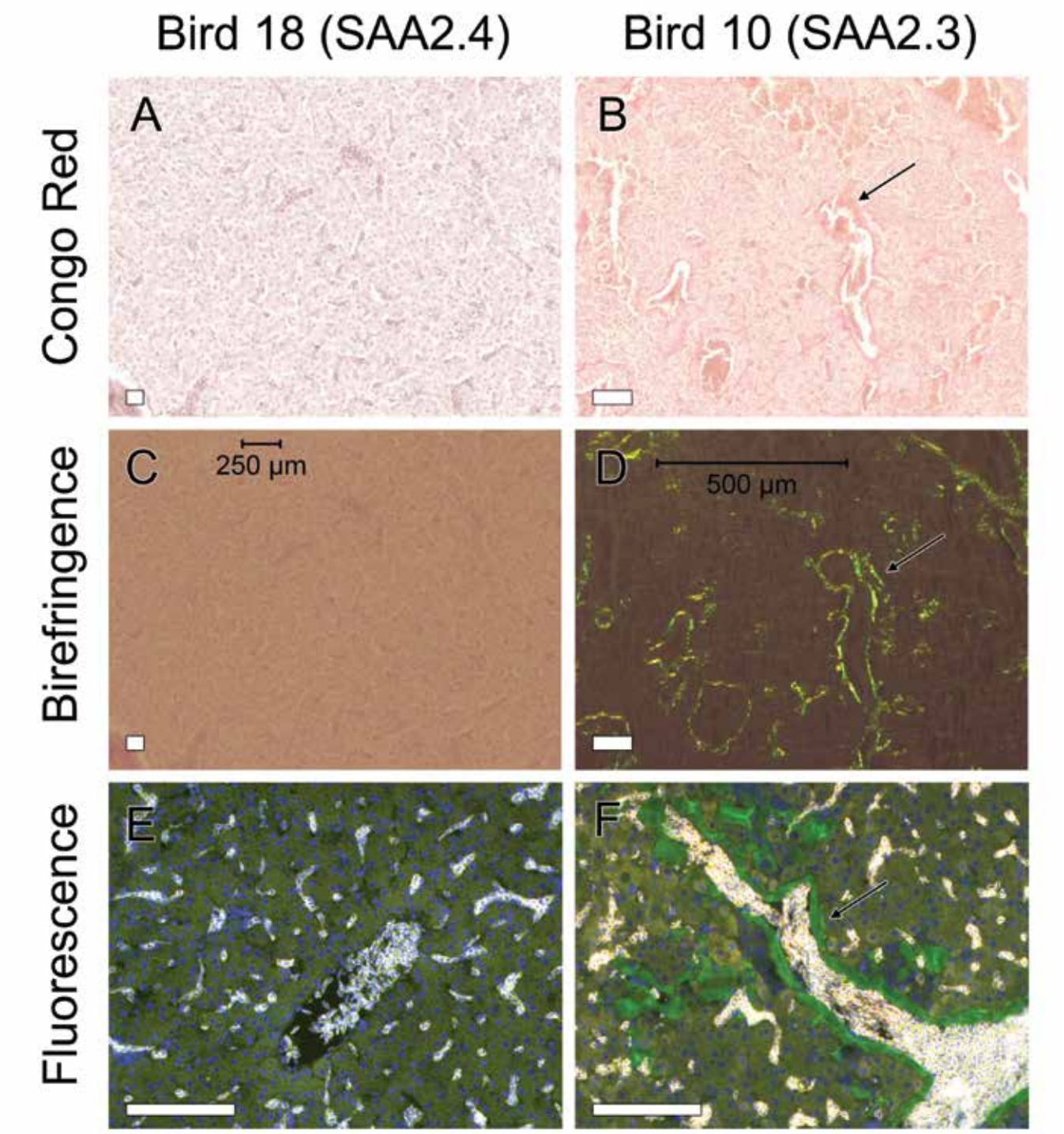


**Figure 1.** Photomicrographs of paraffin-embedded liver sections from representative birds positive (bird 10) or negative (bird 18) for hepatic AA amyloidosis. (A and B) Congo red stain. An arrow marks the distinctive salmon red–orange staining in the perivascular region in bird 10. No distinct staining is seen in bird 18. (C and D) Congo red-stained slides viewed by using polarizing light filter. An arrow marks the green birefringence characteristic for amyloid protein in the perivascular region of bird 10. No birefringence is seen in bird 18. (E and F) Immunofluorescence staining with antiduck amyloid A antiserum. An arrow marks a site of staining in the perivascular region of bird 10, whereas bird 18 shows only minimal staining. White scale bars are 100 µm in all images.

external or internal lesions or abnormalities), except for dorsal feather loss on birds 13 and 15 and a discrete (diameter, 1 to 2 mm), red, subcutaneous mass on bird 18. Hepatic amyloid A deposits were detected in 4 (nos. 6, 13, 10, 11) of these 9 healthy animals (Figure 1). The remaining 6 birds from group 1 were diagnosed with wasting (with or without mycobacteriosis), neoplasia, abscesses, or cholangiohepatitis. Five of these 6 unhealthy animals showed hepatic AA amyloidosis; the remaining bird (no. 9) from this group of 6 had chronic, moderate cholangiohepatitis and *M. genavense* infection but was negative for hepatic AA amyloidosis (Table 1).

Group 2 consisted of 5 birds (nos. 1 through 5) from the same colony as group 1. Group 2 comprised 3 females and 2 males that ranged in age from 2 to 4 y. All birds were wildtype

**Table 1.** Clinical conditions and amyloid A characterization

| Bird no. | Clinical observations | Mycobacterial status | SAA2 variant | Amyloid status[a] |
|---|---|---|---|---|
| Normal body condition | | | | |
| 6 | | — | A1 | PV* +++. P* +/++ |
| 20 | | — | A1 | negative |
| 13 | Feather loss on dorsum | — | A1 | PV +++, P+/++ |
| 15 | Feather loss on dorsum | — | A1 | negative |
| 8 | | + | A2 | negative |
| 14 | | — | A2 | negative |
| 10 | | + | A3 | PV +++, P ++ |
| 11 | | — | A3 | PV +++, P ++ |
| 18 | Small subcutaneous red abdominal mass | — | A4 | negative |
| Wasting or thin | | | | |
| 12 | Pasty vent | — | A2 | P ++++ |
| 5 | Severe, found dead | Acid-fast organisms in tissues | A2 | + group 2 |
| 16 | | + | A4 | PV +/++, P +/++ |
| Neoplasia | | | | |
| 17 | *Hepatic granulosarcoma; wasting* | — | A1 | P +++ |
| 4 | *Hemangiosarcoma (facial mass)* | — | A1 | + group 2 |
| Cholangiohepatitis | | | | |
| 7 | Marked chronic | — | A1 | PV ++, P +++ |
| 9 | Moderate chronic | + | A2 | negative |
| Other illnesses | | | | |
| 1 | Bilateral conjunctivitis, wasting | Acid-fast organisms in tissues | A1 | + group 2 |
| 2 | Pododermatitis, osteomyelitis | — | A1 | + group 2 |
| 19 | Abdominal abscesses | — | A1 | P + |
| 3 | Proventricular ulcer, peritonitis | — | A5 | + group 2 |

*PV, perivascular or within the blood vessels or both; P, parenchyma (Space of Disse).
For group 1 birds, only the liver was removed and examined microscopically. For group 2 birds, see reference 30 for information regarding amyloid severity and distribution.
[a]Amyloid distribution and severity determined according to congophilia, green birefringence by using a polarizing filter, and intensity of fluorescent immunohistochemical staining. Scoring: +, minimal; ++, mild; +++, moderate; and ++++, marked.

for the laboratory-generated DNA construct, except for bird 5 which was transgenic. The birds were euthanized due to various clinical presentations or were found dead and posthumously diagnosed with specific illnesses (Table 1). Systemic AA amyloidosis in 4 of the 5 birds was described in a previous report.[30] Bird 4 from this group was diagnosed with hepatic AA amyloidosis.[30]

## Results

The finding of AA amyloidosis in healthy birds suggests a potential genetic contribution to this disease in zebra finches. To test this idea, we sequenced the SAA2 gene from 20 birds. The genetic diversity was striking, with 18 distinct alleles identified among the 20 animals (Figure 2). Many of the nucleotide changes were synonymous (silent) or located in noncoding DNA and did not change the protein sequence. However, 10 variants produced amino acid substitutions, yielding 5 distinct isotypes of SAA2 that we named SAA2.1 (A1), SAA2.2 (A2), SAA2.3 (A3), SAA2.4 (A4), and SAA2.5 (A5). SAA2.1 was identical to the protein sequence of the NCBI reference allele (Gene ID 100226788), which was derived from a male bird of unknown AA amyloidosis status.[33]

The extent of the nonsynonymous single-nucleotide polymorphisms varied, with SAA2.2 carrying a single amino acid substitution, SAA2.3 having 7 amino acid substitutions and a codon deletion, SAA2.4 harboring 7 amino acid substitutions, and SAA2.5 containing 3 amino acid substitutions (Figure 3). Every SAA2 isotype was associated with at least one bird with AA amyloidosis, some of which showed obvious clinical morbidity or evidence of possible stress, whereas others showed no clear evidence of disease or stress (Table 1). Of most interest to understanding the genetic contribution to disease are the 4 healthy birds (nos. 6, 13, 10, and 11) diagnosed with hepatic amyloid A deposits. Birds 6 and 13 expressed the A1 isotype, whereas birds 10 and 11 expressed the A3 isotype.

The importance of the variants within the population were analyzed by using PROVEAN and PolyPhen2. PROVEAN uses homology and δ alignment scores, whereas PolyPhen2 uses physical and comparative considerations to estimate the relevance of sequence variations to protein function.[1,8] Both programs agreed that most of the amino acid substitutions were not expected to affect the SAA2 protein. However, PROVEAN labeled R52L and V84M as "deleterious," and PolyPhen-2 predicted R52L to be "possibly damaging" and V84M as "probably damaging." The R52L substitution occurred in both the A3 and A4 isotypes, and the A4 isotype contained the V84M substitution (Table 2).

To look more closely at the phylogenetic conservation of the R52 and V84 residues, we aligned zebra finch SAA2 with protein sequences from 35 diverse vertebrate species by using Clustal O. R52 is highly conserved except in mice (*Mus musculus*) and zebrafish (*Danio rerio*), which have lysine residues in this position. Lysine and arginine are basic amino acids with

***SAA2.1***

| Genomic position | G3486 | C3501 | T3971 | C4103 | T4603 | T4659 |
|---|---|---|---|---|---|---|
| aa position | silent | silent | silent | intron | silent | 3'UTR |
| Bird ID | | | | | | |
| **6** | | c/t | c/t | t/t | c/t | -- |
| **7** | | -- | -- | t/t | -- | -- |
| **13** | | c/t | c/t | t/t | c/t | -- |
| **20** | | t/t | c/t | t/t | c/t | -- |
| **1** | | -- | -- | t/t | c/c | -- |
| **4** | | c/t | -- | t/t | c/c | -- |
| **2** | | -- | c/c | t/t | -- | -- |
| **17** | a/a | -- | -- | t/t | c/c | -- |
| **15** | | -- | -- | c/t | c/c | c/c |
| **19** | | -- | -- | c/t | c/c | -- |

***SAA2.2***

| Genomic position | A3387 | T3550 | C4103 | T4603 |
|---|---|---|---|---|
| aa position | 5'UTR | F24V | intron | silent |
| Bird ID | | | | |
| **8** | a/g | t/g | c/t | c/c |
| **9** | -- | g/g | -- | c/c |
| **14** | a/g | g/g | c/t | c/c |
| **12** | -- | t/g | t/t | c/t |
| **5** | -- | t/g | t/t | c/c |

**SAA2.3**

| Genomic position | G3374 | A3398 | A3549 | T3550 | C3593 | A3952 | G3953 | G3961 | T4016 | G4076 | C4103 | A4514 | GGC4517-4519 | G4520 | T4603 | T4659 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| aa position | 5"UTR | 5"UTR | I23M | F24V | A38V | K49S | K49S | R52L | silent | intron | intron | S94G | G95del | V96M | silent | 3"UTR |
| Bird ID | | | | | | | | | | | | | | | | |
| **10** | a/g | a/g | a/g | t/g | c/t | a/g | c/g | g/t | c/t | g/c | c/t | g/a | ---/ggc | a/g | c/c | t/c |
| **11** | a/g | a/g | a/g | t/g | c/t | a/g | c/g | g/t | c/t | g/c | c/t | g/a | ---/ggc | a/g | c/c | t/c |

**SAA2.4**

| Genomic position | A3398 | A3549 | T3550 | C3593 | A3952 | G3953 | G3961 | T4016 | G4054 | G4056 | G4076 | C4103 | T4603 | T4659 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| aa position | 5'UTR | I23M | F24V | A38V | K49S | K49S | R52L | silent | R83K | V84M | intron | intron | silent | 3"UTR |
| Bird ID | | | | | | | | | | | | | | |
| **16** | a/g | a/g | t/g | c/t | a/g | c/g | g/t | c/t | g/a | g/a | g/t | c/t | -- | -- |
| **18** | a/g | a/g | t/g | c/t | a/g | c/g | g/t | c/t | g/a | g/a | g/t | -- | c/c | c/c |

**SAA2.5**

| Genomic position | A3398 | A3549 | T3550 | C3593 | C3624 | C4103 | T4603 |
|---|---|---|---|---|---|---|---|
| aa position | 5"UTR | I23M | F24V | A38V | intron | intron | silent |
| Bird ID | | | | | | | |
| **3** | g/g | g/g | g/g | t/t | t/t | t/t | c/c |

**Figure 2.** DNA mutations and genomic and amino acid positions in the 5 zebra finch SAA2 isotypes. Shading indicates birds diagnosed with AA amyloidosis. Genomic numbering is based on NCBI reference sequence NC_011469.1 (12214080-12208800 *Taeniopygia guttata* isolate Black17 chromosome 5, Taeniopygia_guttata-3.2.4, whole genome shotgun sequence), and amino acid numbering is based on NCBI Gene ID 100226788.

```
SAA2.1 (A1)     MRLWVCIALLSTVLCASADRPRIFDGIIRAGQFVRDAAGG ARDMYRAYKDMREANYKGAD
SAA2.2 (A2)     MRLWVCIALLSTVLCASADRPRIVDGIIRAGQFVRDAAGG ARDMYRAYKDMREANYKGAD
SAA2.3 (A3)     MRLWVCIALLSTVLCASADRPRMVDGIIRAGQFVRDAVGG ARDMYRAYSDMLEANYKGAD
SAA2.4 (A4)     MRLWVCIALLSTVLCASADRPRMVDGIIRAGQFVRDAVGG ARDMYRAYSDMLEANYKGAD
SAA2.5 (A5)     MRLWVCIALLSTVLCASADRPRMVDGIIRAGQFVRDAVGG ARDMYRAYKDMREANYKGAD
                1                      23,24            38           49  52

SAA2.1 (A1)     KYFHARGNYDAARRGPGGAWAARVI SDARENWQSGVSGRGAEDTRLDQEANAWGRSGGDP
SAA2.2 (A2)     KYFHARGNYDAARRGPGGAWAARVI SDARENWQSGVSGRGAEDTRLDQEANAWGRSGGDP
SAA2.3 (A3)     KYFHARGNYDAARRGPGGAWAARVI SDARENWQG-MSGRGAEDTRLDQEANAWGRSGGDP
SAA2.4 (A4)     KYFHARGNYDAARRGPGGAWAAKMI SDARENWQSGVSGRGAEDTRLDQEANAWGRSGGDP
SAA2.5 (A5)     KYFHARGNYDAARRGPGGAWAARVI SDARENWQSGVSGRGAEDTRLDQEANAWGRSGGDP
                                    83,84        94-96

SAA2.1 (A1)     NRYRPKGLPSKY
SAA2.2 (A2)     NRYRPKGLPSKY
SAA2.3 (A3)     NRYRPKGLPSKY
SAA2.4 (A4)     NRYRPKGLPSKY
SAA2.5 (A5)     NRYRPKGLPSKY
                          132
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**Figure 3.** Amino acid sequences for the 5 alleles. Bolding indicates the 18-amino-acid signal sequence. Numbers excluding 1 and 132 correspond to amino acid substitutions compared with the SAA2.1 reference allele. Shading indicates amino acid changes: I23M, F24V. A38V, K49S, R52L, R83K, V84M, S94G, G95del, and V96M. G95 is deleted (shown by a dash) from exon 3 of SAA2.3 and is flanked by S94G and V96M. Breaks in the amino acid sequence signify the start and end of each of the 3 exons.

similar side chains, suggesting that the leucine substitution, which replaces a charged sidechain with a nonpolar sidechain, is likely to affect the structure of SAA2. V84 is conserved in most vertebrate SAA2s but is replaced with alanine in humans and lysine in mice (Figure 4). Whereas the nonpolar alanine found in human SAA2 is chemically like valine, the electrically charged lysine found in mice is quite different from valine. The observation that very different amino acids are tolerated at codon 84

**Table 2.** Functional effects of amino acid sequence variations

| | | PROVEAN | | PolyPhen2 | |
|---|---|---|---|---|---|
| Variants | SNP | Score | Prediction | Score | Prediction |
| A3, A4, A5 | 123M | 0.172 | Neutral | 0.263 | Benign |
| A2, A3, A4, A5 | F24V | –1.059 | Neutral | 0.360 | Benign |
| A3, A4, A5 | A38V | –1.372 | Neutral | 0.023 | Benign |
| A3, A4 | K49S | 1.524 | Neutral | 0.004 | Benign |
| A3, A4 | **R52L** | **–5.587** | **Deleterious** | **0.556** | **Possibly damaging** |
| A4 | R83K | 1.514 | Neutral | 0.002 | Benign |
| A4 | **V84M** | **–2.695** | **Deleterious** | **0.999** | **Probably damaging** |
| A3 | S94G | 0.142 | Neutral | 0.004 | Benign |
| A3 | G95del | 0.232 | Neutral | — | Not applicable |
| A3 | V96M | 0.428 | Neutral | 0.307 | Benign |

According to PROVEAN, variants with a score ≤ –2.5 are considered deleterious; variants with a score > –2.5 are considered neutral. According to PolyPhen2, scores range from 0.0 (tolerated) to 1.0 (deleterious), with 0.0–0.15 considered benign, 0.15–1.0 as possibly damaging, and 0.85–1.0 more confidently predicted to be damaging (Ion Reporter Software, Thermofisher Scientific). PolyPhen2 does not evaluate deletions.

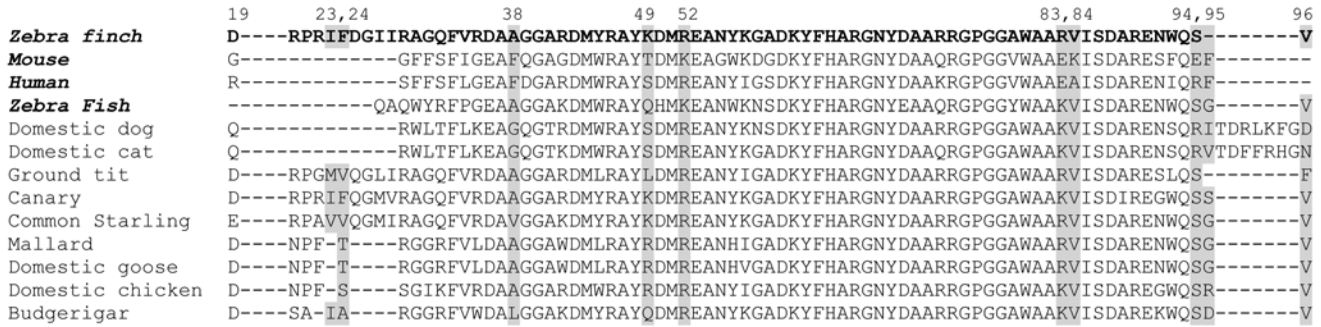


**Figure 4.** CLUSTAL Omega multiple-sequence alignment representing a 13-species subset from 36 aligned vertebrate species. The 18-amino-acid leader sequences have been removed. UMMS zebra finch sequence is bolded and aligned from amino acid 19 through 96. UMMS zebra finch colony SNP and G95del (designated by a dash) are shaded in gray with amino acid sequence number superscripts. Aligned amino acids for zebra finch SNP and G95 codon deletion are shaded gray. The names of species that are discussed in the text (zebra finch, *Taeniopygia guttata*; mouse, *Mus musculus*; human, *Homo sapiens*; and zebra fish, *Danio rerio*) are italicized and bolded.

raises questions regarding the pathogenicity of methionine at this position.

In addition to 7 amino acid substitutions, the A3 isotype carries a codon deletion, which has the potential to be disruptive. In a porcine case report, splenic and renal AA amyloidosis were found in a pig that expressed an SAA2 sequence with 2 amino acid deletions (W2 and L3) and an L6V polymorphism.[16] In the cited case, fibrillization studies revealed that amyloid fibrils from the variant porcine SAA2 converted the wild-type SAA2 protein into amyloid fibrils.[16] In addition, the PROVEAN predictor rated the 2 deletions as "deleterious" and the L6V substitution as "neutral." The G95 codon that is deleted in zebra finch A3 is not highly conserved in vertebrates (Figure 4), suggesting that this codon probably is not critical and was predicted by PROVEAN to be benign (Table 2).

The 2 amino acids predicted to be most damaging were found in alleles with numerous other substitutions (Figure 3). In addition to the R52L substitution, the A3 allele had 6 additional substitutions and a codon deletion, and the A4 allele had both the R52L and V84M substitutions as well as 5 additional altered residues. The cumulative effects of multiple mutations may be more deleterious than that predicted by the web-based algorithms. A study of Japanese patients with rheumatoid arthritis correlated the prevalence of AA amyloidosis with SNPs in SAA1. The study found that disease risk might be better assessed by using multiple SNPs.[2]

In addition to changes in coding potential of the various SAA2 alleles, silent mutations and changes in nontranslated sequences were extensive within the population (Figure 2). Although the importance of these changes is difficult to assess, they could have important effects on splicing and protein translation.[7,17,18] These types of changes are associated with human disease phenotypes,[7,29] and a –13T polymorphism in the 5′ flanking region of the SAA1 allele has been linked to the development of AA amyloidosis in Japanese and Caucasian patients with rheumatoid arthritis.[23]

## Discussion

In summary, the *SAA2* gene in our zebra finch colony was polymorphic and heterogeneous, with almost as many alleles as birds. We conclude that the A3 variant shows the strongest potential for amyloidogenicity. In the future, this association could be explored by selective breeding and in vitro experiments. In addition, the wide genetic diversity within the gene allows for exploration of protective alleles as several birds with amyloidogenic comorbidities did not develop AA amyloidosis.

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