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Original Research

Natural Progression of Canine Glycogen Storage Disease Type IIIa

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Glycogen storage disease type IIIa (GSD IIIa) is caused by a deficiency of glycogen debranching enzyme activity. Hepatomegaly, muscle degeneration, and hypoglycemia occur in human patients at an early age. Long-term complications include liver cirrhosis, hepatic adenomas, and generalized myopathy. A naturally occurring canine model of GSD IIIa that mimics the human disease has been described, with progressive liver disease and skeletal muscle damage likely due to excess glycogen deposition. In the current study, long-term follow-up of previously described GSD IIIa dogs until 32 mo of age (n = 4) and of family-owned GSD IIIa dogs until 11 to 12 y of age (n = 2) revealed that elevated concentrations of liver and muscle enzyme (AST, ALT, ALP, and creatine phosphokinase) decreased over time, consistent with hepatic cirrhosis and muscle fibrosis. Glycogen deposition in many skeletal muscles; the tongue, diaphragm, and heart; and the phrenic and sciatic nerves occurred also. Furthermore, the urinary biomarker Glc_4, which has been described in many types of GSD, was first elevated and then decreased later in life. This urinary biomarker demonstrated a similar trend as AST and ALT in GSD IIIa dogs, indicating that Glc_4 might be a less invasive biomarker of hepatocellular disease. Finally, the current study further demonstrates that the canine GSD IIIa model adheres to the clinical course in human patients with this disorder and is an appropriate model for developing novel therapies.

Abbreviations: CCR, curly-coated retriever; CPK, creatine phosphokinase; GSD IIIa, glycogen storage disease type IIIa; Glc_4, Glcα1-6Glcα1-4Glcα1-4Glc.

Glycogen storage disease type IIIa (GSD IIIa; OMIM, 232400) is an autosomal recessive disorder caused by mutations in the glycogen debranching enzyme gene (*AGL*), leading to various clinical signs. The tissues mainly affected are liver, heart, and skeletal muscle. Clinical manifestations include hypoglycemia, elevated serum concentrations of liver and muscle enzymes, hepatomegaly, growth retardation, muscle weakness, cardiac hypertrophy with arrhythmia risk, polycystic ovaries and neuropathy.[15,17,29] Current treatments are mainly symptomatic and are not curative. The most frequently used therapies are dietary, such as providing uncooked corn starch to prevent hypoglycemia at young ages and high-protein diets, which have been shown to reverse the extent of cardiomyopathy associated with GSD IIIa.[7,8,30,37] In addition, the use of medium-chain triglycerides has shown positive therapeutic effects in patients with GSD Ia and GSD IIIa.[11,22] However, dietary therapies do not prevent the long-term complications of GSD IIIa, including hepatic cirrhosis, hepatocellular adenoma, hepatocellular carcinoma, cardiomyopathy, neuropathy, and myopathy.[31]

An appropriate animal model is necessary to test novel therapies and address the long-term effects of GSD IIIa. Recently a mouse model for GSD III has been described that may prove beneficial in testing new therapies.[19] However, the limitations of mouse models include a short lifespan that curtails the study of the long-term effects of novel treatments. In addition, a large animal model often mimics human disease more closely than do mouse models, as occurs in GSD type Ia dog models, which exhibit lactic acidosis similar to human patients, a characteristic that mouse models of GSD Ia lack.[16] Therefore a naturally occurring large animal model for GSD IIIa may be more effective in terms of the development of new treatments than are available mouse models.

GSD IIIa (OMIA, 001577) has been reported to occur in curly-coated retriever dogs (CCR) and is caused by a naturally occurring homozygous frameshift mutation in exon 32 that leads to the deletion of 126 amino acids at the C-terminus of glycogen debranching enzyme.[12,40] The dogs in these previous studies proved to have abnormalities similar to those seen in humans affected with the disorder, namely progressive glycogen accumulation in muscle and liver, elevated liver and muscle enzymes (ALP, AST, creatine phosphokinase [CPK], and ALT), and eventual liver fibrosis. However, these animals were not followed beyond 16 mo of age in the earlier studies.[12,40] The goal of the current study is to provide biochemical follow-up on these animals and analyze more extensively other tissues and organs involved in GSD IIIa in the dog model. A brief analysis of the naturally high protein diets of GSD IIIa dogs, as well as the effects of an increased protein diet in 2 dogs for the last few months of life, is included.

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We also include the analysis of a urinary biomarker, Glcα1–6Glcα1– 4Glcα1–4Glc (Glc_4), which is a breakdown product of glycogen by α-amylase and neutral α-1,4-glucosidase.[32] Elevated levels of Glc_4 have been found in urine from patients with GSD types II, III, IV, VI, and IX and may correlate with disease advancement.[1,18,24,32] To our knowledge, Glc_4 has not been evaluated previously in dogs; we therefore here evaluated the utility of Glc_4 as a biomarker of canine GSD IIIa. A correlation of Glc_4 levels with liver enzyme concentrations in blood might indicate a role of Glc_4 as a less-invasive biomarker for determining the advancement of liver disease in human and canine patients.

Materials and Methods

Animal use. The 6 CCR dogs with GSD IIIa analyzed here were previously reported until 16 and 42 mo of age (Figure 1).[12,40] Dogs E and J were female littermates and analyzed until 32 mo of age, dog B was a male dog followed to 20 mo of age, and dog D was a female dog analyzed until 19 mo of age. Dog J received treatment with an adeno-associated viral vector expressing acid α-glucosidase at 12 mo of age; analysis of liver biopsy from the dog 5 wk after vector injection revealed a low level of transduction (0.14 vector genomes per cell) and no obvious increase of acid α-glucosidase expression. There were no significant changes in the subsequent biochemical data of dog J, and these data were therefore included, but her tissues were not analyzed (Figure 1). All aspects of this project were performed with approval of the Duke University IACUC. Dogs were maintained according the standards in the *Guide for the Care and Use of Laboratory Animals*[23] at Duke University (Durham, NC), an AAALAC-accredited institution. Dog diets were the same as previously reported,[40] except for dog B as reported in the clinical summary, and dogs E and J were switched to a diet with increased protein at 29 mo of age (Figure 1). The high-protein diet included 1 can of Science Diet Puppy Gourmet Chicken Entrée (Hill's Pet Nutrition, Topeka, KS) and 3.5 cups of EVO Turkey and Chicken Formula Dry Dog Food (Natura Pet Products, Fremont, NE) daily; this diet equated to approximately 9.4 g protein/kg daily, whereas the previous diet was about 6.6 to 7.4 g protein/kg daily. Blood was collected from either the cephalic or jugular veins from each dog monthly and sent to a commercial laboratory for a routine panel of biochemical tests (Antech, Diagnostic Laboratories, Cary, NC) as described previously.[40] Urine was collected free catch from trays placed under the individual dog runs or by cystocentesis. Urinary Glc_4 was measured at the Duke Biochemical Genetics Laboratory by using techniques described previously.[1]

Biochemical data and medical history of the older GSD IIIa dogs were from the dogs identified as CCR 1 and CCR 2 in a previous study[12] (Figure 1). Medical history and laboratory data were provided by permission of the owners from their veterinarians (Dr Laura Snyder, Franklinville Animal Hospital, Franklinville, NJ, and Vetco Animal Hospital, Tabernacle, NJ, and Dr Linda Mathias, Point Breeze Veterinary Clinic, Pittsburgh, PA). Surgery notes from CCR 1 were provided from staff veterinarians (Garden State Veterinary Specialists, Tinton Falls, NJ).

Urine biomarker reference range. Urine from an unaffected dog was obtained from a wild-type CCR mixed-breed dog at 2, 3, 4 and 6 mo of age and contained 1.71 ± 1.58 mmol Glc_4 per mol CN (mean ± 1 SD). Additional urine samples were analyzed from 4 other unaffected dogs (age, 2 to 10 y). The overall mean of these combined data was 1.16 ± 0.51 mmol Glc4 per mol CN. The normal reference range for Glc_4 was established as mean ± 2 SD.

Tissue collection. Dogs were euthanized at 19 to 32 mo of age by using intravenous administration of Euthasol (Virbac Animal Health, Fort Worth, TX) according to the manufacturer's directions. Necropsy was performed immediately thereafter, and multiple tissues were collected for analysis. Each tissue was either preserved in 10% neutral buffered formalin, 3% glutaraldehyde in 0.2 M sodium cacodylate buffer (Electron Microscopy Science, Hatfield, PA), or flash-frozen after being covered in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA). Tissues were then embedded and sectioned for histopathologic analysis as described.[40] Specimens were stained using Richardson–periodic acid–Schiff stain, hematoxylin and eosin, or trichrome stains. Liver and muscle glycogen concentrations were quantified from frozen tissues as previously described.[40]

Results

Glycogen accumulation in multiple tissues. Multiple tissues in 3 of the 6 GSD IIIa affected dogs in the current study were analyzed histologically (Figure 1). Glycogen deposition was found in the diaphragm, gastrocnemius, tongue, and quadriceps in all 3 dogs (Figure 2 A through C).[14] Glycogen deposits were present within cardiomyocytes of the older dog, dog E, but were not noted in the 2 younger dogs (Figure 2 D). The sciatic and phrenic nerves demonstrated glycogen accumulation in Schwann cell cytoplasm of the younger dogs (Figure 2 E and F). Interestingly, similar to recent findings in GSD II patients, some glycogen deposition was present within extraocular muscles in dog E (Figure 3).[26,27]

Changes in hepatic and muscle glycogen content over time. Hepatic glycogen content decreased over time from 180 ± 59 µmol glucose/g tissue previously reported at 16 mo of age (n = 3)[40] to an average of 98 ± 5 µmol glucose/g tissue at 19 mo of age (n = 2) and 120 ± 10 µmol glucose/g tissue in 2 different dogs at 32 mo of age. Muscle glycogen was 93 ± 4 µmol glucose/g tissue at 19 mo of age (n = 2) and 121 ± 29 µmol glucose/g tissue in 2 dogs at 32 mo of age, which had decreased from a previously reported average of 168 µmol glucose/g tissue at 16 mo of age (n = 3).[40] In summary, hepatic glycogen appeared to decrease in conjunction with decreased ALT and AST activities beginning at about 16 mo of age (Figure 4 A and B).[40] Muscle glycogen decreased later in life, beginning at about 19 mo of age (data not shown), along with a decrease in the activity of the muscle enzyme CPK (Figure 4 D). The decrease in hepatic and muscle glycogen was confirmed histologically in serial biopsies from dog E, at 16 and 32 mo of age (Figure 5). In addition, increased hepatic fibrosis leading to cirrhosis was demonstrated over time histologically in dog E (Figure 5 A through D).

Biochemistry and urinary biomarkers. A previous study demonstrated a gradual increase in liver enzyme activities up to 16 mo of age.[40] Follow-up of the same animals (n = 2 to 4) revealed that these enzymes subsequently decreased over time. ALT (normal range, 12 to 118 U/L) peaked between 12 to 20 mo, ranging from 476 to 688 U/L, but decreased as low as 204 ± 9.2 U/L after 28 mo of age (Figure 4 A). AST (normal range, 15 to 66 U/L) peaked between 12 to 19 mo, ranging between 235 to 534 U/L, but decreased to as low as 118 ± 47.4 U/L after 24 mo of age (Figure 4 B). ALP (normal range, 5 to 131 U/L) was slightly more variable than ALT and AST and appeared to peak between 12 to 23 mo, range between 309 to 554 U/L, but decrease as low as 162.5 ±

	Treatment	Age at euthanasia	Data and tissues analyzed in current study	Previously published data
Dog B (male)	Diet change to i/d canine gastrointestinal health diet (Hill's) after 15 mo of age; intermittent treatment with proton pump inhibitors for vomiting	20 mo	Blood chemistries; urine Glc_4 (Fig. 4); urolith, diaphragm, gastrocnemius,[a] tongue,[a] quadriceps,[a] heart,[a] sciatic nerve,[a] and phrenic nerve[a,b]	Blood chemistries, muscle and liver glycogen content, and histopathologic images until 16 mo of age in references 39 and 40 as UT1
Dog D (female)	Intermittent treatment with proton pump inhibitors and i/d canine gastrointestinal health diet (Hill's) for vomiting; intermittent treatment with antibiotics and analgesics for wounds in lower extremity from approximately 11 to 14 mo of age	19 mo	Blood chemistries; urine Glc_4 (Fig. 4); diaphragm (Figure 2 A), gastrocnemius (Figure 2 B), tongue,[a] quadriceps,[a] heart,[a] phrenic nerve (Figure 2 E), and sciatic nerve (Figure 2 F)[b]	Blood chemistries, muscle and liver glycogen content and gross and histopathologic images until 16 mo of age in references39 and 40 as UT2; tongue histopathology and brief clinical history in reference 14
Dog E (female)	Higher protein diet at 29 mo of age	32 mo	Blood chemistries; urine Glc_4 (Fig. 4); diaphragm,[a] gastrocnemius,[a] tongue (Figure 2 C), heart (Figure 2 D), extraocular muscles (Figure 3), liver (Figure 5 A through D), and quadriceps (Figure 5 E and F)[c]	Blood chemistries and muscle and liver glycogen content until 16 mo of age in reference 40
Dog J (female)	AAV–GAA at 12 mo of age; higher protein diet at 29 mo of age	32 mo	Blood chemistries and urine Glc_4 (Figure 4)[d]	Blood chemistries and muscle and liver glycogen content until 12 mo of age in reference 40
CCR 1 (female)	Analgesics for lameness andweakness at approximately 12 y of age	12 y	Clinical history and blood chemistries (Figure 6)	Initial clinical history described in reference 12
CCR 2 (female)	S-adenosylmethionine and milk thistle beginning at approximately 6 y of age; intermittent analgesics for hindlimb lameness	11 y	Clinical history and blood chemistries (Figure 6)	Initial clinical history described in reference 12

Figure 1. Summary of GSD IIIa CCR dogs used in this study. a, Tissues analyzed but not shown here; b, extraocular muscles were not collected at the time of necropsy and thus were unavailable for analysis; c, nerves were not collected at the time of necropsy and therefore were unavailable for analysis; d, tissues not analyzed due to AAV-GAA treatment, no significant differences noted on biochemical data.

62.9 U/L after 25 mo of age (Figure 4 C). CPK (normal range, 59 to 895 U/L) was variable among dogs during 13 to 22 mo of age and rose as high as 3561 ± 3673.4 U/L at 18 mo of age but then decreased to normal or near-normal ranges after 24 mo of age (Figure 4 D). GGT was mildly elevated, reaching a maximum of 16 U/L (normal range, 1 to 12 U/L) in a few dogs at various time points after 12 mo of age (Figure 4 E). All other biochemical parameters, including serum cholesterol, triglycerides, glucose, bilirubin, albumin, BUN, and creatinine were within normal ranges for the lifetime of the animals.

The serum liver enzymes analyzed for the 2 older GSD IIIa-affected CCR demonstrated similar trends. ALT and AST peaked between 2 to 3 y of age, getting as high as 1180 U/L and 905 U/L, respectively, but decreased to less than 290 U/L and 166 U/L, respectively, after 6.5 y of age in CCR 2 (Figure 6 A and B). In comparison, ALT and AST in CCR 1 stayed a little more constant throughout her lifetime, with a gradual rise after 6 y of age (Figure 6 A and B). ALP mainly remained elevated throughout the lifetimes of the 2 older dogs but increased further, to as high as 1080 U/L, after 8 y of age (Figure 6 C). CPK was elevated only at 1 y of age in CCR 2 and then remained at near-normal or within normal limits throughout the rest of her life (Figure 6 D). CCR1 had a single report of a CPK level higher than 30,000 U/L, at 3.3 y of age (not shown), but otherwise CPK was within normal limits throughout her lifetime (Figure 6 D). GGT mostly remained within normal limits for both dogs but was elevated to 18 U/L after 8 y of age (Figure 6 E). Serum cholesterol (normal range, 92 to 324 mg/dL) likewise increased to as high as 360 mg/dL after 7 y of age in both older dogs (Figure 6 F). Triglycerides remained within normal limits throughout the lifetime of CCR 1 and 2 (not shown). These findings support data from human patients, with increased liver fibrosis over time according to decreased AST and ALT activities and are consistent with increased cholestasis in light of the increases in cholesterol, ALP, and GGT levels over time.

The urinary biomarker Glc_4 was demonstrated to correlate positively with serum AST and ALT in GSD IIIa dogs (R = 0.76 and 0.83, respectively; $P < 0.001$). Over time, urine Glc_4 showed a similar rise and increase to those of the liver enzymes, with peak Glc_4 values in GSD IIIa-affected dogs ($n = 2$ to 4) between 10 to 23 mo of age, and values approaching the calculated normal range beginning at 24 mo of age (Figure 4 F).

Hepatomegaly. Findings at necropsy in 2 of the 32-mo-old dogs and a single 20-mo-old dog revealed liver weights averaging 5% ± 1% of body weight. The normal dog liver weighed 3.38% of body weight, on average, in a study of 91 dogs.[21] The exact liver weight was not determined in younger dogs; however, serial abdominal palpation and examination of livers during laparotomies for liver biopsies suggest that hepatomegaly is more pronounced at younger ages in GSD IIIa CCR dogs.

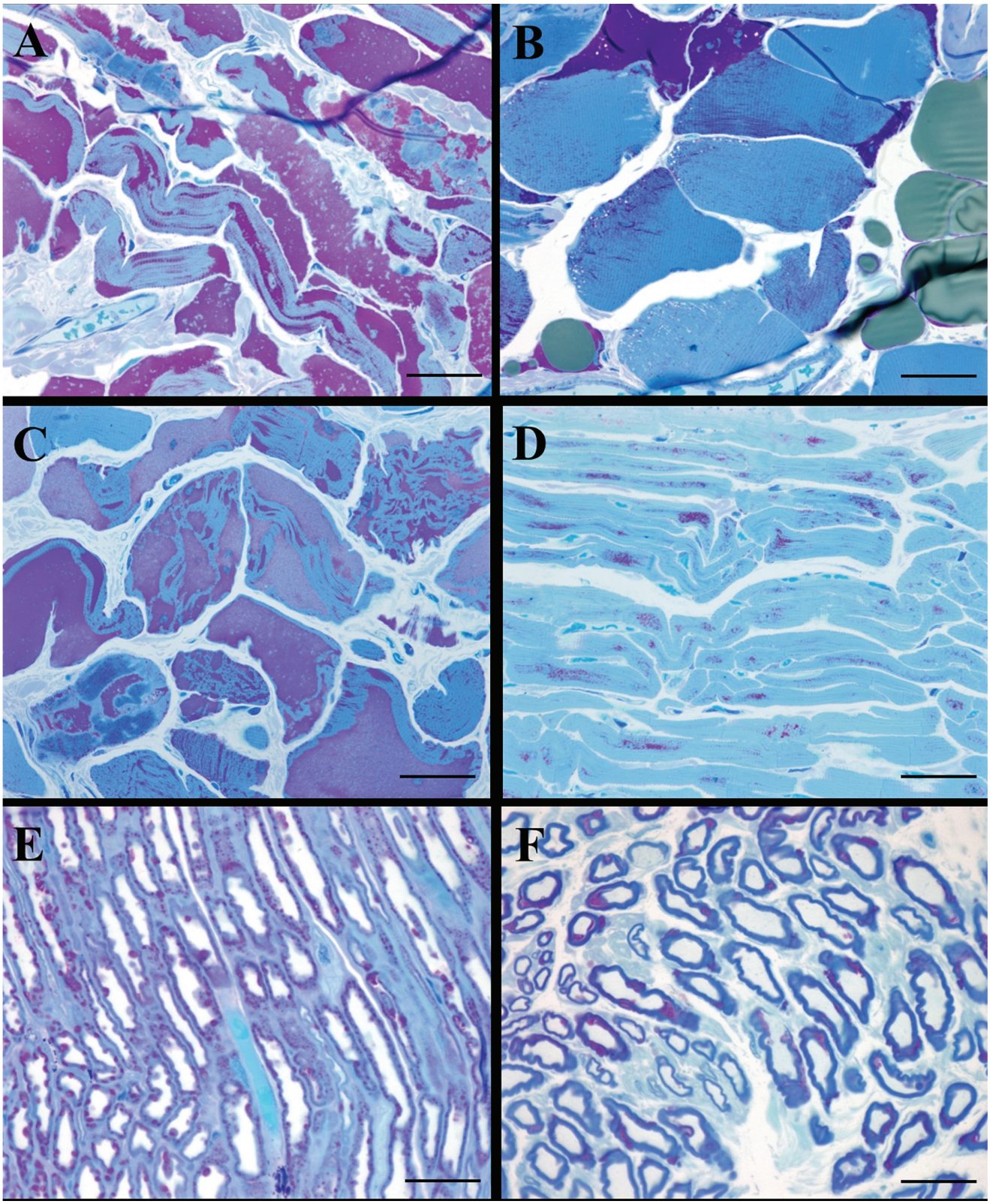

Figure 2. Glycogen deposition in tissues from dogs with GSD IIIa. Glycogen accumulation (purple) is present in the cytoplasm in prominent lake-like formations, which disrupt the normal myofibrillar architecture of skeletal myocytes in the (A) diaphragm, (B) gastrocnemius, and (C) tongue. Glycogen deposition also is present within (D) cardiomyocytes and in the Schwann cell cytoplasm of both the (E) phrenic and (F) sciatic nerves. Epoxy resin, Richardson–periodic acid–Schiff stain; magnification: 400× (A through D; scale bar, 50 µm), 600× (E and F; scale bar, 25 µm). Tissues in panels A, B, E, and F are from dog D at 19 mo of age; and samples in panels C and D are from dog E at 32 mo of age.

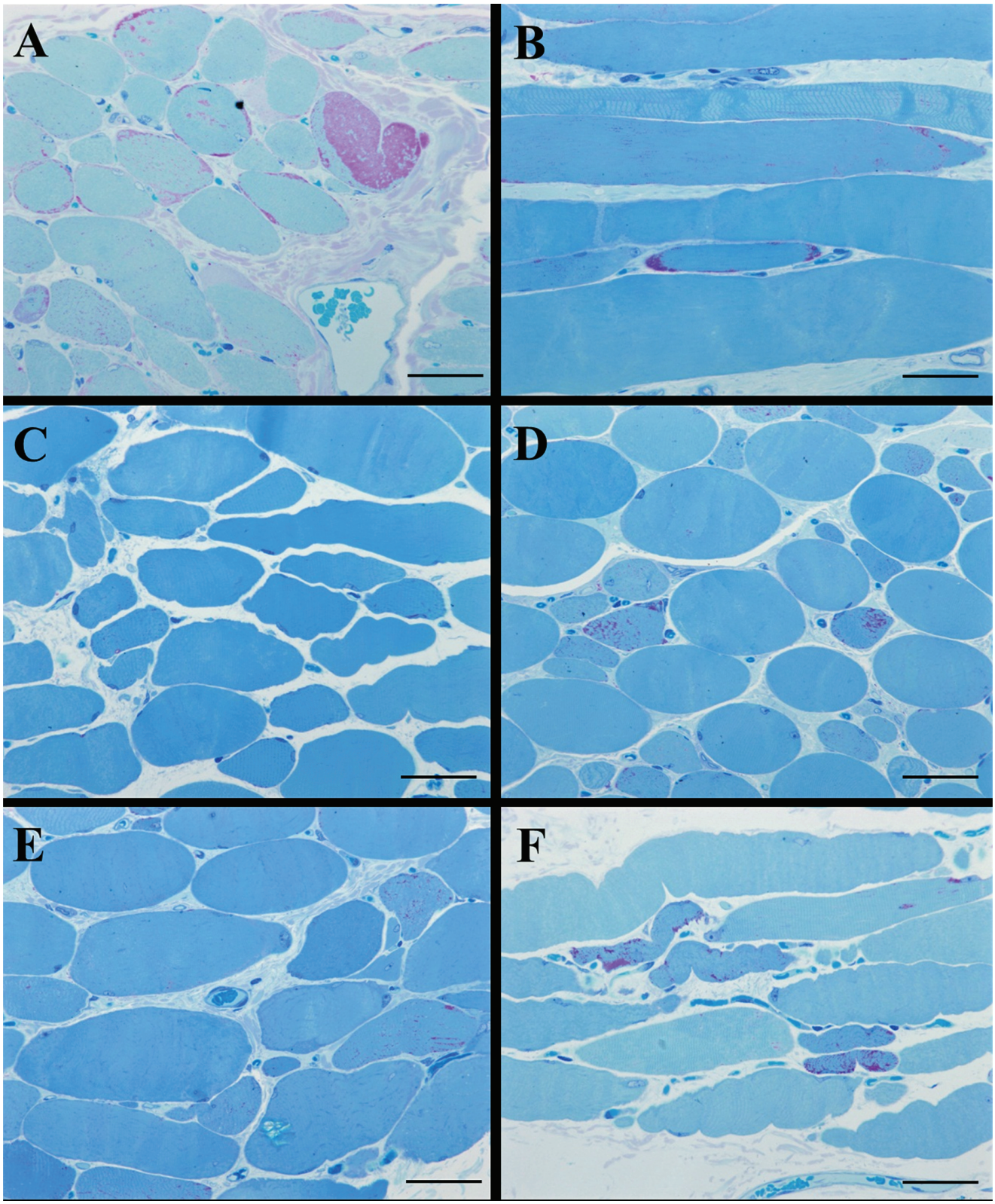

Figure 3. Minimal glycogen deposition in extraocular muscles. Glycogen deposition (purple) affected only a few of the myocytes from extraocular muscles of dog E at 32 mo of age, including the (A) dorsal oblique, (B) ventral oblique, (C) lateral rectus, (D) medial rectus, (E) ventral rectus, and (F) retractor bulbi. Epoxy resin, Richardson–periodic acid–Schiff stain; magnification, 400× (scale bar, 50 µm).

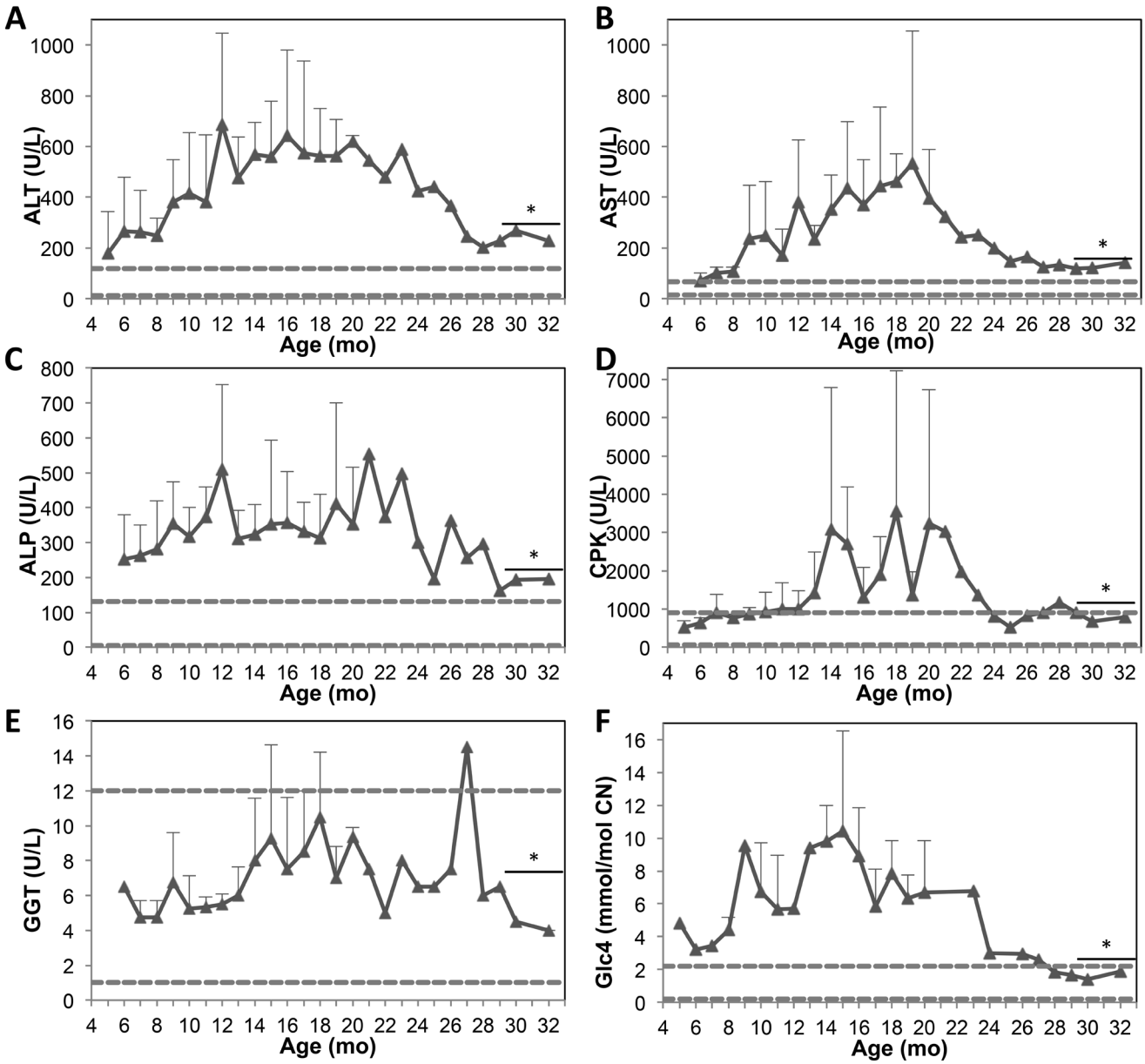

Figure 4. Serum enzyme activities and concentrations of the urinary biomarker Glc_4 in CCR with GSD IIIa. Serum concentrations of (A) ALT, (B) AST, (C) ALP, (D) CPK, and (E) GGT in CCR with GSD IIIa and sampled from 5 to 32 mo of age (n = 4 from 5 to 19 mo; n = 3 [dogs B, E, and J] at 20 mo, and n = 2 [dogs E and J] from 21 to 32 mo). Enzyme activities from the same dogs at earlier time points were previously reported.[40] (F) Urinary concentrations of biomarker Glc_4 in GSD IIIa-affected dogs from 4 to 32 mo of age (n = 2 to 4). Data are given as mean ± 1 SD; dashed lines represent normal reference values. The * indicates initiation of the high-protein diet (9.4 g/kg daily).

Clinical findings in GSD IIIa CCR dogs. All GSD IIIa CCR dogs in the laboratory setting (n = 4) remained clinically stable throughout their lifetime, with normal activity, attitude, and appetites, with the exception of 2 dogs. One dog, dog B, maintained a good appetite, activity, and attitude but chronically vomited or regurgitated his food approximately once or twice every 1 to 2 wk. His diet was switched to i/d Canine Gastrointestinal Health Diet (Hill's Pet Nutrition) and periodically was treated with proton pump inhibitors (ranitidine, famotidine), but the mild vomiting or regurgitating persisted throughout his lifetime (Figure 1). At necropsy, dog B had diffuse nodular fibrosis indicative of cirrhosis throughout all liver lobes.

Dog D presented with lethargy and ascites at 19 mo of age, with a week-long history of slightly decreased appetite and activity and occasional vomiting. A brief clinical history of dog D was provided previously.[14] Necropsy and histopathology revealed severe cirrhosis of the liver, which likely led to the observed ascites and eventual need for euthanasia (see reference 39 for gross and histopathologic images of liver).

Although 32-mo-old dogs E and J did not exhibit overt clinical signs of disease during their lifetime, biochemical data, findings at necropsy and histologic examination of their tissues indicated that disease was apparent. Dog E had histologic evidence of liver cirrhosis at 32 mo of age (Figure 5 B and D).

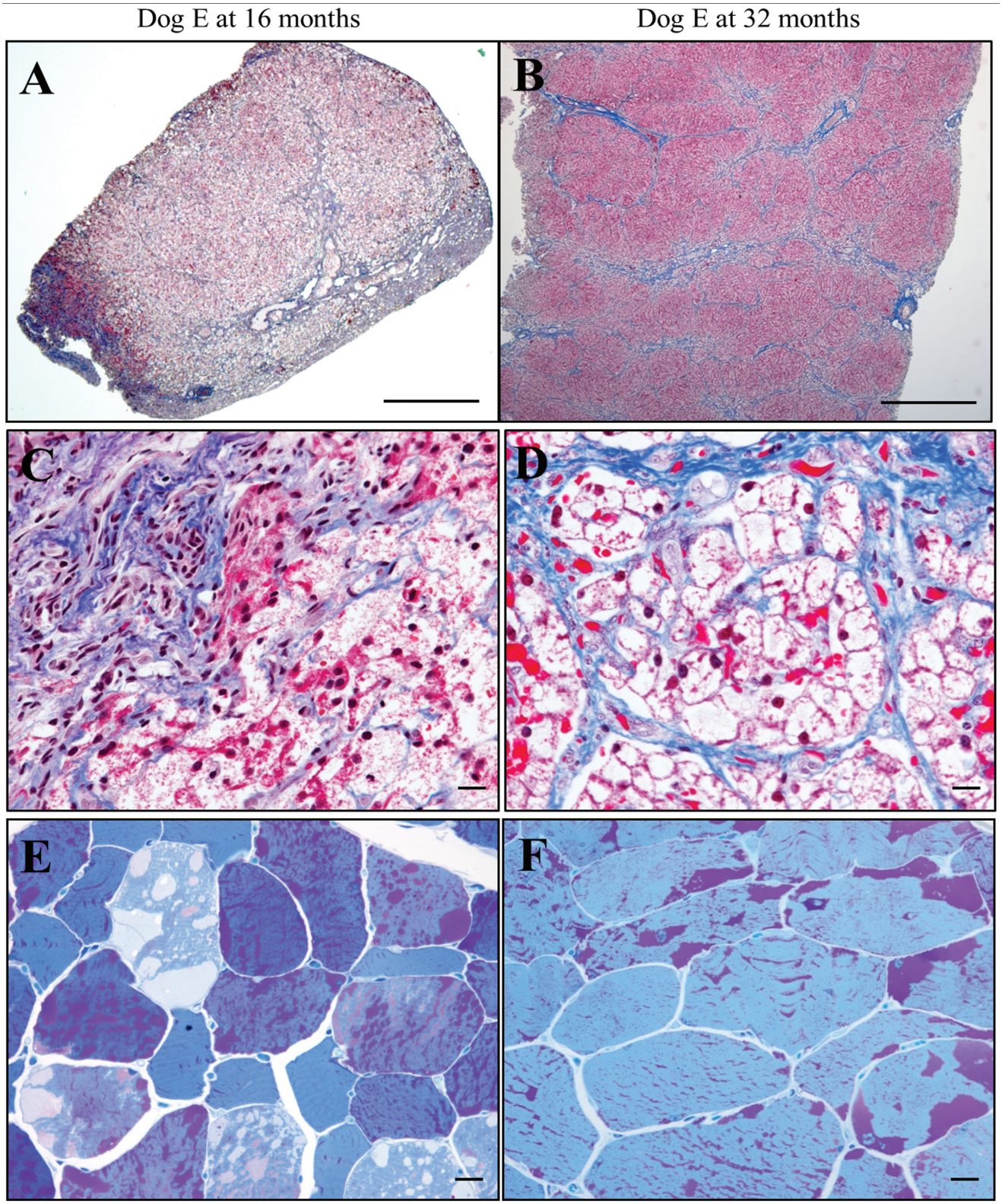

Figure 5. Decrease in glycogen and increase in fibrosis over time in the liver and muscle of dog with GSD IIIa (dog E). Liver at (A, C) 16 mo of age demonstrates more glycogen accumulation and less fibrosis than does liver from the same dog at (B, D) 32 mo of age. (B, D) Note the micronodule formation in the liver at 32 mo of age. Trichrome stain; magnification: 20× (scale bar, 1 millimeter; A and B); 400× (scale bar, 20 µm; C and D). Quadriceps at (E) 16 mo of age demonstrates more glycogen within myocytes than do those at (F) 32 mo of age. Richardson–periodic acid–Schiff stain; magnification, 400× (scale bar, 20 µm; E and F).

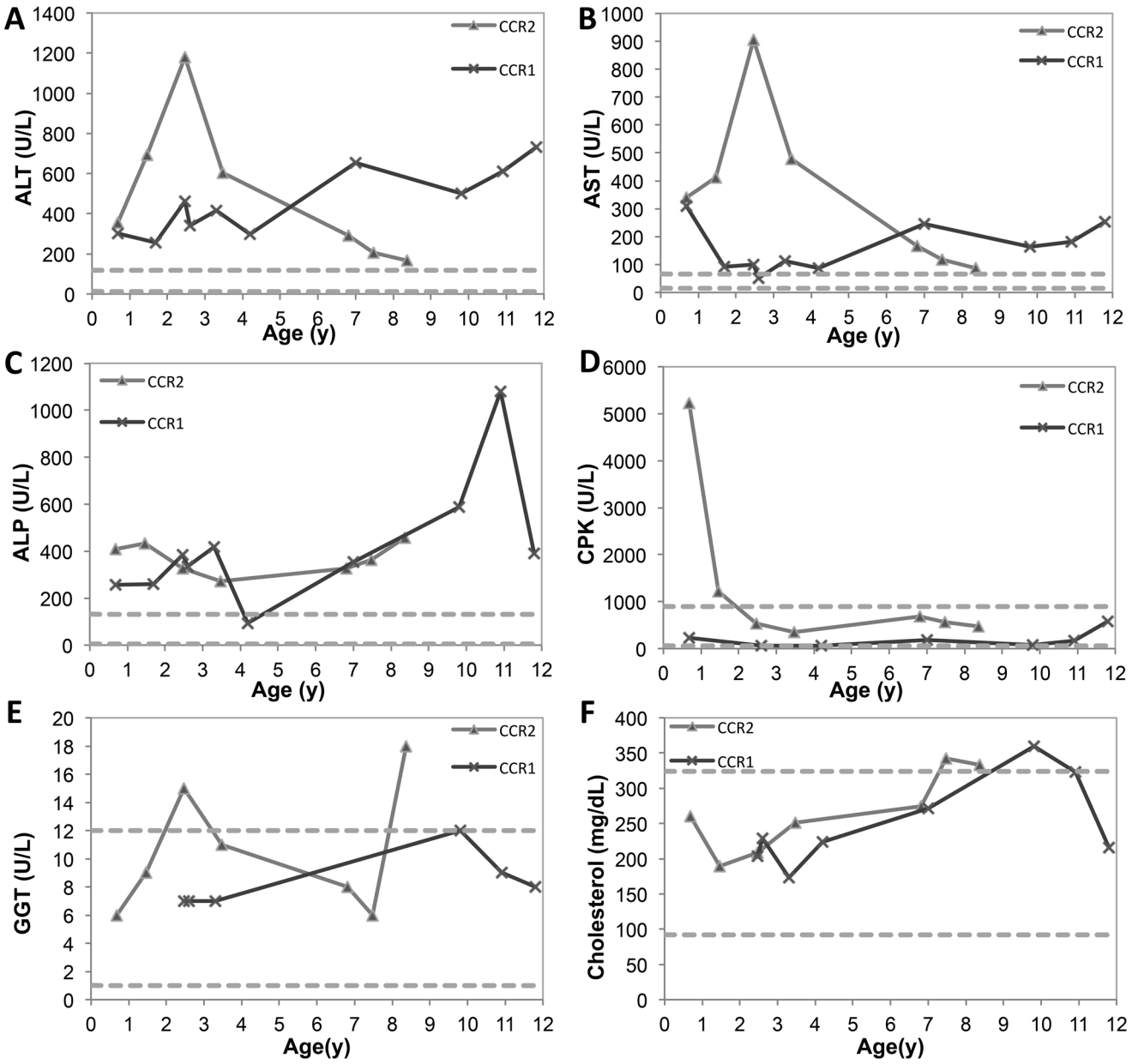

Figure 6. Serum enzyme activities and lipid concentrations in older CCR dogs with GSD IIIa. Serum (A) ALT, (B) AST, (C) ALP, (D) CPK, (E) GGT, and (F) cholesterol concentrations at 6 mo to 12 y of age. Early data from these same dogs was presented previously.[12] Dashed lines indicate normal reference values.

The older dogs described as CCR 1 and 2 remained clinically normal throughout much of their lifetime, with occasional episodes of hypoglycemia during times of increased physical activity that resolved with glucose therapy and rest. Both displayed decreased activity and lameness or soreness of limbs at about 8 to 11 y of age due to either arthritis or muscle weakness. At 10 y of age, CCR 2 developed a palpable mass on her flank. A fine-needle aspirate was consistent with sarcoma but was not evaluated further. The mass continued to grow, and her activity and appetite decreased; she ultimately was euthanized without necropsy, according to her owner's request. CCR 1 became increasingly lethargic and presented as anemic with hemoabdomen at 12 y of age. During an exploratory laparotomy, hemorrhagic masses were found on a grossly enlarged and abnormal liver, as well as on the spleen, intestines, and peritoneum. The dog was euthanized, and no histopathology or necropsy was performed, according to the owner's request.

Urolithiasis. An incidental finding at necropsy of dog B was small granules within the penile prepuce, but none were observed in the bladder or kidneys. Analysis by the Minnesota Urolith Center (St Paul, MN) revealed that the granules were composed of ammonium urate. No clinical signs consistent with urolithiasis were noted in this or any other GSD IIIa dog. A previous case series reported that 2 of 5 human GSD III patients had kidney stones but did not describe their composition.[34] Patients with GSD Ia have been reported to have stones consisting of calcium oxa-

late and calcium phosphate likely due to hypocitraturia.[4,28] In the Duke GSD Clinic, the team has documented at least 2 patients with GSD III (in a cohort of 32 patients) with recurrent urolithiasis or particles in nephrons. The citrate level was normal in one patient (the stone composition was unknown) and low in the second patient (stone due to hypocitraturia). According to anecdotal reports, urine organic-acid analysis has revealed hypocitraturia in additional patients without a history of kidney stones.

Discussion

Canine models have demonstrated their utility in determining the natural progression of genetic disorders and in the development and testing of novel therapies. For example, the GSD IIIa CCR model has shed light on previously unrecognized complications of GSD IIIa, including dysphagia and lingual weakness,[14] and has demonstrated the efficacy of small molecule therapy with rapamycin.[39] The current study sheds light on various similarities between canine and human patients with long-term GSD IIIa. Some of the important similarities include those regarding biochemical abnormalities, multiple tissue involvement, and clinical findings (Figure 7). Findings in the canine model but not currently recognized in human patients, such as glycogen deposition in the diaphragm and extraocular muscles, should be followed closely to aid in clinical work-up of patients with GSD IIIa.

Decreases in ALT, AST, ALP, and CPK have been demonstrated in human GSD IIIa patients over time;[5,20,34] here it is shown that GSD IIIa CCRs display a similar trend, as noted to 32 mo in the laboratory setting and to 12 y in the home setting. These results and the histologic evidence of liver cirrhosis are consistent with the theory that increased hepatic fibrosis ultimately will cause decreases in the liver enzymes ALT and AST. This study represents the first time that clinical laboratory and full necropsy-based histologic correlations have been possible, thus providing insight into the disease progression. According to the findings from the GSD IIIa dogs, decreases in hepatic glycogen content and serum liver enzyme activities are actually signs of progressive hepatic disease rather than improvement. These findings are consistent with those reported previously.[6,9]

Reports of increased AST:ALT ratio as a noninvasive method to diagnose liver fibrosis—ratios greater than 0.87 being indicative of moderate liver fibrosis[36]—seem to correlate loosely with biochemical data collected from the GSD IIIa CCR dogs. For example, dog D, which demonstrated the most advanced stage of liver cirrhosis at necropsy among the dogs studied, had AST:ALT ratios of 0.7 to 2.2 after 12 mo of age. AST:ALT ratios might be useful in GSD IIIa patients to monitor hepatic fibrosis and eventual cirrhosis.

In adult dogs, ALP is most often elevated in cases of cholestasis but may also be increased due to bone destruction or remodeling or to corticosteroid stimulation.[33] However, the combination of increases in ALP, GGT, and cholesterol after 7 y of age in CCR 1 and 2 and the increases in biliary sludge found at necropsy of a few GSD IIIa CCR dogs are suggestive of cholestasis. There is limited evidence that increases in bone ALP may be due to low bone mineral density in GSD III patients,[3] but radiographic imaging that was performed on a few animals provided no clinical evidence of this situation in GSD IIIa CCR dogs (data not shown). ALP is often elevated due to the bone isoform of ALP in young, growing dogs but is generally not elevated to the concentrations demonstrated here.[33] Given the variability of ALP in GSD IIIa CCR dogs, ALP may not be as good a prognostic indicator of GSD III liver disease as are ALT and AST. Analysis of the specific ALP isoforms might provide insight into the cause of ALP increases in GSD IIIa.

Although one GSD IIIa dog demonstrated glycogen accumulation within cardiomyocytes, no other heart-associated abnormalities like those described previously in human GSD IIIa patients were detected.[2] The minimal heart involvement in GSD IIIa dogs may be due to the already high protein diet that is provided for GSD III dogs. One group[37] reported 6 g/kg protein daily as a high-protein diet for GSD III human patients, and another[7] described changes with 3 g/kg daily, whereas in the current study, about 7 g/kg protein daily was the normal diet for GSD III dogs. Furthermore, the change to a higher protein diet (9.4 g protein/kg daily) may have helped to decrease the ALP and CPK levels in dogs E and J after 28 mo of age (Figure 4 C and D). These data provide supporting evidence that a high-protein diet might prevent or slow the progression of skeletal and cardiac disease in GSD III patients. However, long-term diet changes with negative controls need to be performed to provide more conclusive data to support this hypothesis.

The presence of glycogen within nerves of GSD IIIa CCR dogs suggests that a portion of the muscle weakness associated with GSD IIIa might be neuropathic in nature. This hypothesis is consistent with findings of decreased nerve conduction velocities, median nerve entrapment, and distal myopathies observed in GSD IIIa patients.[10,13,25,35,38] Not all of the GSD IIIa CCR dogs studied displayed clinical signs of exercise intolerance or muscle weakness, but this apparent inconsistency is most likely due to the laboratory setting. CCR dogs are bred for hunting and are accustomed to vigorous exercise outdoors; despite having large enclosures and adequate play time, exercise intolerance likely was not displayed due to the decrease in physical activity compared with the level the breed normally encounters. This notion is supported by the data from the older GSD IIIa dogs in the home setting, which had bouts of hypoglycemia associated with prolonged activity, but hypoglycemia was never exhibited in the laboratory setting.

In summary, the current study demonstrates the GSD IIIa CCR dog as a viable model to aid in increased understanding of GSD IIIa in humans and to drive improved clinical treatment strategies. However, we acknowledge the challenges to using large animal models for development of novel therapeutics of genetic disorders. Some of these challenges include the variations between species in terms of drug metabolism, the costs and long-term commitment associated with sustaining a research dog colony while trying to maintain an adequate gene pool, the ability to conduct large-scale studies with many animals, and the variation in disease progression among species or individuals. In light of these challenges, there are many advantages of using animal models in finding cures for diseases such as GSD III. Due to their shorter lifespan compared with that of humans, dogs offer a greater opportunity to study the natural progression of disease and therefore the effects of novel treatments in a shorter timeframe than that available in traditional human clinical trials. In addition, the use of animal models provides a controlled setting in which to decrease or eliminate environmental or other confounding factors that might play a role in the advancement of disease. At this time, the canine GSD IIIa model appears to be a highly valuable model in replicating many aspects of the human disease and can serve an important role in finding a cure for GSD III.

Clinical findings in CCR dogs with GSD IIIa	Similar findings in human patients with GSD
Increased ALT, AST, and CPK levels that decreased over time in 5 dogs	Increased ALT, AST, and CPK that decreased over time[5,9,13,20,34]
Increased AST:ALT ratio associated with hepatic cirrhosis in 1 dog	Not described
Increased ALP in 6 dogs	Increased ALP[5,34]
Increased urinary biomarker Glc_4 that correlated with decreases in ALT and AST over time in 4 dogs	Increased urinary biomarker Glc_4[18,24,32]
Glycogen accumulation with hepatomegaly that decreased over time in 4 dogs	Glycogen accumulation with hepatomegaly that decreased over time[5]
Progressive liver fibrosis and cirrhosis in 3 dogs	Liver cirrhosis[6,9,31]
Masses in liver, spleen, peritoneum, and intestines in 1 older dog	Hepatocellular carcinoma[6,9,31]
Glycogen accumulation in skeletal muscles in 3 dogs: quadriceps, gastrocnemius, and diaphragm	Glycogen accumulation in skeletal muscles: quadriceps, gastrocnemius by biopsy;[10,25] glycogen accumulation in calf muscles detected through nuclear MRI[38]
Possible muscle weakness after 8 y of age in 2 dogs	Neuropathy and myopathy associated with increased age[13,20,35,38]
Glycogen accumulation in diaphragms of 3 dogs	Not described
Minimal glycogen accumulation in extraocular muscles in 1 dog	Ptosis and vacuolar myopathy in extraoculuar muscles with glycogen accumulation in cornea, retina, and lens in patients with GSD II[26,27]
Glycogen deposition in cardiomyocytes in a 32-month-old dog	Glycogen accumulation in the atrioventricular node and in smooth muscle cells of intramyocardial arteries[2]
Glycogen accumulation in tongues of 3 dogs	Dysarthria or dysphagia in 3 patients and fatty infiltration and fibro–fatty expansion in the lingual septum detected through MRI[14]
Glycogen accumulation in sciatic and phrenic nerves in 2 dogs	Glycogen accumulation in the sural nerve (n = 1); decreased nerve conduction velocities;[10,35] selective glycogen accumulation by Schwann cells in unmyelinated nerves[25]
Glycogen accumulation in adipocytes[40]	Glycogen accumulation in adipocytes (PSK and SA, current study)
Hypoglycemic episodes during activity in 2 dogs	Hypoglycemia commonly described[15]
Polycystic ovaries in 1 dog (unpublished observation, current study)	Polycystic ovaries associated with hyperinsulinemia in patients with GSD I and III[17,29]
Ammonium urate urolithiasis in 1 dog	2 of 5 patients with kidney stones;[34] 2 patients with recurrent nephrourolithiasis in Duke GSD Clinic; hypocitraturia with no history of stones in other GSD III patients (anecdotal reports; PSK and SA, current study)

Figure 7. Summary of clinical findings in CCR with GSD IIIa compared with human patients with GSD.

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References

1. **An Y, Young SP, Hillman SL, Van Hove JL, Chen YT, Millington DS.** 2000. Liquid chromatographic assay for a glucose tetrasaccharide, a putative biomarker for the diagnosis of Pompe disease. Anal Biochem **287:**136–143.
2. **Austin SL, Proia AD, Spencer-Manzon MJ, Butany J, Wechsler SB, Kishnani PS.** 2012. Cardiac pathology in glycogen storage disease type III. JIMD Rep **6:**65–72.
3. **Cabrera-Abreu J, Crabtree NJ, Elias E, Fraser W, Cramb R, Alger S.** 2004. Bone mineral density and markers of bone turnover in patients with glycogen storage disease types I, III, and IX. J Inherit Metab Dis **27:**1–9.
4. **Chen YT.** 1991. Type I glycogen storage disease: kidney involvement, pathogenesis, and its treatment. Pediatr Nephrol **5:**71–76.
5. **Coleman RA, Winter HS, Wolf B, Chen YT.** 1992. Glycogen debranching enzyme deficiency: long-term study of serum enzyme activities and clinical features. J Inherit Metab Dis **15:**869–881.
6. **Cosme A, Montalvo I, Sanchez J, Ojeda E, Torrado J, Zapata E, Bujanda L, Gutierrez A, Arenas I.** 2005. [Type III glycogen storage disease associated with hepatocellular carcinoma] Gastroenterol Hepatol **28:**622–625. [Article in Spanish].
7. **Dagli AI, Zori RT, McCune H, Ivsic T, Maisenbacher MK, Weinstein DA.** 2009. Reversal of glycogen storage disease type IIIa-related cardiomyopathy with modification of diet. J Inherit Metab Dis **32 Suppl 1:** 103–106.
8. **Das AM, Lucke T, Meyer U, Hartmann H, Illsinger S.** 2010. Glycogen storage disease type 1: impact of medium-chain triglycerides on metabolic control and growth. Ann Nutr Metab **56:**225–232.
9. **Demo E, Frush D, Gottfried M, Koepke J, Boney A, Bali D, Chen YT, Kishnani PS.** 2007. Glycogen storage disease type III—hepatocellular carcinoma a long-term complication? J Hepatol **46:**492–498.
10. **DiMauro S, Hartwig GB, Hays A, Eastwood AB, Franco R, Olarte M, Chang M, Roses AD, Fetell M, Schoenfeldt RS, Stern LZ.** 1979. Debrancher deficiency: neuromuscular disorder in 5 adults. Ann Neurol **5:**422–436.
11. **El-Gharbawy AH, Arnold GL, Perrott-Taylor N, Hughley T, Long K, Vockley J, Kishnani PS.** 2014. Optimizing metabolic control of glycogen storage disease type 3 (GSD3): potential role for medium-chain triglycerides (Mct). Mol Genet Metab **111:**284–285.
12. **Gregory BL, Shelton GD, Bali DS, Chen YT, Fyfe JC.** 2007. Glycogen storage disease type IIIa in curly-coated retrievers. J Vet Intern Med **21:**40–46.

13. **Hobson-Webb LD, Austin SL, Bali DS, Kishnani PS**. 2010. The electrodiagnostic characteristics of glycogen storage disease type III. Genet Med **12:**440–445.
14. **Horvath JJ, Austin SL, Jones HN, Drake EJ, Case LE, Soher BJ, Bashir MR, Kishnani PS.** 2012. Bulbar muscle weakness and fatty lingual infiltration in glycogen storage disorder type IIIa. Mol Genet Metab **107:**496–500.
15. **Kishnani PS, Austin SL, Arn P, Bali DS, Boney A, Case LE, Chung WK, Desai DM, El-Gharbawy A, Haller R, Smit GP, Smith AD, Hobson-Webb LD, Wechsler SB, Weinstein DA, Watson MS.** 2010. ACMG practice guidelines. Glycogen storage disease type III diagnosis and management guidelines. Genet Med.**12:**446–463.
16. **Kishnani PS, Faulkner E, VanCamp S, Jackson M, Brown T, Boney A, Koeberl D, Chen YT.** 2001. Canine model and genomic structural organization of glycogen storage disease type Ia (GSD Ia). Vet Pathol **38:**83–91.
17. **Lee PJ, Patel A, Hindmarsh PC, Mowat AP, Leonard JV.** 1995. The prevalence of polycystic ovaries in the hepatic glycogen storage diseases: its association with hyperinsulinism. Clin Endocrinol (Oxf). **42:**601–606.
18. **Lennartson G, Lundblad A, Sjoblad S, Svensson S, Ockerman PA.** 1976. Quantitation of a urinary tetrasaccharide by gas chromatography and mass spectrometry. Biomed Mass Spectrom **3:**51–54.
19. **Liu KM, Wu JY, Chen YT.** 2014. Mouse model of glycogen storage disease type III. Mol Genet Metab **111:**467–476.
20. **Lucchiari S, Santoro D, Pagliarani S, Comi GP.** 2007. Clinical, biochemical and genetic features of glycogen debranching enzyme deficiency. Acta Myol.**26:**72–74.
21. **Evans HE, DeLahunta A.** 2013. Miller's anatomy of the dog, 4th ed. St Louis (MO): Elsevier Saunders.
22. **Nagasaka H, Hirano K, Ohtake A, Miida T, Takatani T, Murayama K, Yorifuji T, Kobayashi K, Kanazawa M, Ogawa A, Takayanagi M.** 2007. Improvements of hypertriglyceridemia and hyperlacticemia in Japanese children with glycogen storage disease type Ia by medium-chain triglyceride milk. Eur J Pediatr **166:**1009–1016.
23. **Institute for Laboratory Animal Research.** 2011. Guide for the care and use of laboratory animals, 8th ed. Washington (DC): National Academies Press.
24. **Oberholzer K, Sewell AC.** 1990. Unique oligosaccharide (apparently glucotetrasaccharide) in urine of patients with glycogen storage diseases. Clin Chem **36:**1381.
25. **Powell HC, Haas R, Hall CL, Wolff JA, Nyhan W, Brown BI.** 1985. Peripheral nerve in type III glycogenosis: selective involvement of unmyelinated fiber Schwann cells. Muscle Nerve **8:**667–671.
26. **Prakalapakorn SG, Proia AD, Yanovitch TL, DeArmey S, Mendelsohn NJ, Aleck KA, Kishnani PS.** 2014. Ocular and histologic findings in a series of children with infantile Pompe disease treated with enzyme replacement therapy. J Pediatr Ophthalmol Strabismus **51:**355–362.
27. **Prater SN, Banugaria SG, DeArmey SM, Botha EG, Stege EM, Case LE, Jones HN, Phornphutkul C, Wang RY, Young SP, Kishnani PS**. 2012. The emerging phenotype of long-term survivors with infantile Pompe disease. Genet Med **14:**800–810.
28. **Scales CD Jr, Chandrashekar AS, Robinson MR, Cantor DA, Sullivan J, Haleblian GE, Leitao VA, Sur RL, Borawski KM, Koeberl D, Kishnani PS, Preminger GM.** 2010. Stone forming risk factors in patients with type Ia glycogen storage disease. J Urol **183:**1022–1025.
29. **Sechi A, Deroma L, Lapolla A, Paci S, Melis D, Burlina A, Carubbi F, Rigoldi M, Di Rocco M.** 2012. Fertility and pregnancy in women affected by glycogen storage disease type I—results of a multicenter Italian study. J Inherit Metab Dis **36:**83–89.
30. **Sentner CP, Caliskan K, Vletter WB, Smit GP.** 2011. Heart failure due to severe hypertrophic cardiomyopathy reversed by low-calorie, high-protein dietary adjustments in a glycogen storage disease type IIIa patient. JIMD Rep **5:**13–16.
31. **Siciliano M, De Candia E, Ballarin S, Vecchio FM, Servidei S, Annese R, Landolfi R, Rossi L.** 2000. Hepatocellular carcinoma complicating liver cirrhosis in type IIIa glycogen storage disease. J Clin Gastroenterol **31:**80–82.
32. **Sluiter W, van den Bosch JC, Goudriaan DA, van Gelder CM, de Vries JM, Huijmans JG, Reuser AJ, van der Ploeg AT, Ruijter GJ.** 2012. Rapid ultraperformance liquid chromatography–tandem mass spectrometry assay for a characteristic glycogen-derived tetrasaccharide in Pompe disease and other glycogen storage diseases. Clin Chem **58:**1139–1147.
33. **Syakalima M, Takiguchi M, Yasuda J, Hashimoto A.** 1997. The age-dependent levels of serum ALP isoenzymes and the diagnostic significance of corticosteroid-induced ALP during long-term glucocorticoid treatment. J Vet Med Sci **59:**905–909.
34. **Talente GM, Coleman RA, Alter C, Baker L, Brown BI, Cannon RA, Chen YT, Crigler JF Jr, Ferreira P, Haworth JC, Herman GE, Issenman RM, Keating JP, Linde R, Roe TF, Senior B, Wolfsdorf JI.** 1994. Glycogen storage disease in adults. Ann Intern Med **120:**218–226.
35. **Ugawa Y, Inoue K, Takemura T, Iwamasa T.** 1986. Accumulation of glycogen in sural nerve axons in adult-onset type III glycogenosis. Ann Neurol **19:**294–297.
36. **Ustundag Y, Bilezikci B, Boyacioglu S, Kayatas M, Odemir N.** 2000. The utility of AST:ALT ratio as a noninvasive demonstration of the degree of liver fibrosis in chronic HCV patients on long-term haemodialysis. Nephrol Dial Transplant **15:**1716–1717.
37. **Valayannopoulos V, Bajolle F, Arnoux JB, Dubois S, Sannier N, Baussan C, Petit F, Labrune P, Rabier D, Ottolenghi C, Vassault A, Broissand C, Bonnet D, de Lonlay P.** 2011. Successful treatment of severe cardiomyopathy in glycogen storage disease type III with D,L-3-hydroxybutyrate, ketogenic and high-protein diet. Pediatr Res **70:**638–641.
38. **Wary C, Nadaj-Pakleza A, Laforet P, Claeys KG, Carlier R, Monnet A, Fleury S, Baligand C, Eymard B, Labrune P, Carlier PG.** 2010. Investigating glycogenosis type III patients with multiparametric functional NMR imaging and spectroscopy. Neuromuscul Disord **20:**548–558.
39. **Yi H, Brooks ED, Thurberg BL, Fyfe JC, Kishnani PS, Sun B.** 2014. Correction of glycogen storage disease type III with rapamycin in a canine model. J Mol Med (Berl) **92:**641–650.
40. **Yi H, Thurberg BL, Curtis S, Austin S, Fyfe J, Koeberl DD, Kishnani PS, Sun B.** 2012. Characterization of a canine model of glycogen storage disease type IIIa. Dis Model Mech **5:**804–811.