Characterization of a canine model of glycogen storage disease type IIIa

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Running Title: Canine model of GSD IIIa

Key words: Glycogen storage disease type IIIa, canine model, debranching enzyme
ABSTRACT

Glycogen storage disease type IIIa (GSD IIIa) is an autosomal recessive disease caused by deficiency of glycogen debranching enzyme (GDE) in liver and muscle. The disorder is clinically heterogeneous and progressive, and there is no effective treatment. Previously a naturally occurring dog model for this condition was identified in curly-coated retrievers (CCR). The affected dogs carry a frame-shift mutation in the GDE gene and have no detectable GDE activity in liver and muscle. We characterized in detail the disease expression and progression in eight dogs from age 2 to 16 months. Monthly blood biochemistry revealed elevated and gradually increasing serum alanine transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) activities; serum creatine phosphokinase (CPK) activity exceeded normal range after 12 months. Analysis of tissue biopsy specimens at 4, 12, and 16 months revealed abnormally high glycogen contents in liver and muscle of all dogs. Fasting liver glycogen content increased from 4 months to 12 months, but dropped at 16 months possibly caused by extended fibrosis; muscle glycogen content continually increased with age. Light microscopy revealed significant glycogen accumulation in hepatocytes at all ages. Liver histology showed progressive, age-related fibrosis. In muscle, scattered cytoplasmic glycogen deposits were present in most cells at 4 months, but large, lake-like accumulation developed by 12 and 16 months. Disruption of the contractile apparatus and fraying of myofibrils was observed in muscle at 12 and 16 months by electron microscopy. In conclusion, the CCR dogs are an accurate model of GSD IIIa that will improve our understanding of the disease progression and allow opportunities to investigate treatment interventions.
INTRODUCTION

Mutations in glycogen debranching enzyme (GDE) gene cause glycogen storage disease type III (GSD III), resulting in accumulation of cytoplasmic glycogen in liver and muscle, the two major tissues for glycogen metabolism (Illingworth and Cori, 1952; Illingworth et al., 1956). GDE is a bifunctional protein having two distinct enzymatic activities: 1,4-α-D-glucan:1,4 α-D-glucan 4-α-D-glycosyltransferase (EC 2.4.1.25) and amylo-1,6-glucosidase (EC 3.2.1.33) (Taylor et al., 1975; Nakayama et al., 2001). Together with glycogen phosphorylase, GDE is responsible for complete degradation of cytoplasmic glycogen. More than 80% of GSD III patients have debranching enzyme deficiencies in both liver and muscle (type IIIa), while most of the rest manifest only liver involvement (type IIIb) (Van Hoof and Hers, 1967; Kishnani et al., 2010).

General clinical manifestations of GSD IIIa include hepatomegaly, fasting hypoglycemia, hyperlipidemia, growth retardation, and variable myopathy and cardiomyopathy. However, disease phenotypes vary widely in patients most likely caused by different GDE mutations specific to individual families on different genetic and environmental backgrounds (Hobson-Webb et al., 2010; Kishnani et al., 2010). Liver symptoms often appear in childhood and typically improve after puberty, but liver cirrhosis and hepatic adenoma or hepatocellular carcinoma have been reported in some cases (Haagsma et al., 1997; Labrune et al., 1997; Siciliano et al., 2000; Cosme et al., 2005; Demo et al., 2007). Progressive myopathy is the major cause of morbidity in GSD IIIa patients. Muscle weakness is usually not a prominent feature during childhood but can progress with age, rendering some patients wheel chair bound in their third or fourth decade of lives (Momoi et al., 1992; Lucchiari et al., 2007; Kishnani et al., 2010). PAS-positive glycogen storage can be observed in adult patients along with distorted myofiber structures (Kim et al., 2008; Schoser et al., 2008). Glycogen deposition in cardiac muscle has
been recognized since 1968 and ventricular hypertrophy is common in GSD IIIa patients (Pearson, 1968; Moses et al., 1989; Lee et al., 1997). Patients with cardiac involvement are at risk of heart failure and life threatening arrhythmias, yet the actual incidence is relatively low (Miller et al., 1972; Moses et al., 1989; LaBarbera et al., 2010). Consistent with liver and muscle damages, laboratory tests also show elevated serum alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), and creatine phosphokinase (CPK) activities (Coleman et al., 1992; Lee et al., 1995; Lucchiari et al., 2007; Karwowski et al., 2011).

As the search for an effective treatment for GSD IIIa is ongoing and the pathophysiology of the disease and mechanisms of clinical variability are not well understood, an appropriate animal model that mimics human disease is needed. Recently GSD IIIa was identified in curly-coated retrievers (CCR) (Gregory et al., 2007). The affected dogs carry a frame-shift mutation predicting deletion of the C-terminal 126 amino acids of GDE and resulting in a GSD IIIa phenotype. Investigation on two affected dogs showed no detectable GDE enzyme activity in liver and muscle. Analysis of liver biopsies revealed severe glycogen accumulation but there was no evidence of inflammation or fibrosis in the liver. PAS-positive glycogen deposits were observed in a skeletal muscle biopsy taken from one of them at age 14 months (Gregory et al., 2007). However a thorough characterization of this model and of disease characteristics has not been performed. We have established a breeding colony of the CCR dogs to better understand the phenotype, and to allow for better understanding of disease progression. In this report, we describe this canine GSD IIIa model in extensive biochemical and histological details.
RESULTS

1. Serum biochemistry

Monthly routine serum biochemistry panels of 8 affected dogs revealed gradually increasing liver enzyme activities in these dogs. ALT (normal range 12-118 U/L) increased from 144±36 U/L at 2 months to a peak of more than 700 U/L at 15 months (Fig 1A). AST (normal range 15-66 U/L) was slightly higher than normal before 6 months (70-80 U/L), then gradually increased to 283±67 U/L at 13 months, followed by a jump to nearly 600 U/L at 14 months (Fig 1B). The average value of ALP (normal range 5-131 U/L) fluctuated between 200 and 400 U/L with a trend of increasing with age (Fig 1C). The CPK level (normal range 59-895 U/L) in the tested dogs was in the normal range before age 10 months, became slightly above normal from 10 to 12 months, and then continually increased to above 2000 U/L at 16 months (Fig 1D).

Triglycerides (Fig 1E) and cholesterol (Fig 1F) concentrations were normal in all dogs throughout the study. All other parameters including glucose, bilirubin, albumin, urea nitrogen, and creatinine were within normal ranges. There is no difference in the growth curves of affected dogs and their normal littermates.

2. Liver and muscle glycogen contents

Liver and muscle biopsies were performed on GSD IIIa dogs after overnight fasting at ages of 4, 12, and 16 months. Liver glycogen content (Fig 2A) was 209±47 µmol glucose/g tissue at 4 months of age, more than 4 folds of that found in a normal dog (~47 µmol glucose/g). The value increased to 269±44 µmol glucose/g at 12 months and dropped to 180±59 µmol glucose/g at 16 months. The decrease of liver glycogen content from age 12 to 16 month was likely due to large scale replacement of hepatocytes by fibrous tissue as described later, and correlates well with the observed accelerated increase of serum ALT and AST activities in the same time frame (Fig 1A,
B). Gradually increasing muscle glycogen content was observed in all dogs. As shown in Fig 2B, muscle glycogen contents were 81±11, 104±12, and 168±49 μmol glucose/g tissue at ages 4, 12, and 16 months, respectively, compared to ~ 23 μmol glucose/g tissue of a normal dog. The damaging effect of high glycogen content on muscle is evidenced by the sharply increased serum CPK and AST activities after 12 months (Fig 1) and by histopathological analysis described later.

3. Gross and histological appearance of liver

Liver and muscle are two major tissues affected by glycogen storage. High glycogen turnover rates in liver (Magnusson et al., 1994) predispose this organ to glycogen deposition even at young ages in GSD IIIa. At age 4 months when the first liver biopsy was performed, the livers were enlarged and fragile with relatively smooth surfaces in all dogs (n=4). At 12 months, the livers were severely enlarged and firmer, with small nodules scattered on the surface (n=4). At 16 months, the severely and diffusely enlarged livers were partially or fully involved with large nodules and cirrhosis (n=3). Histological analysis of liver specimens revealed marked glycogen accumulation in hepatocytes at all ages and a gradual disturbance of hepatocellular organization with age. As shown in Fig 3, H&E stained paraffin sections (Panels A through C) exhibited the typical vacuolated appearance of glycogen filled hepatocytes; in HRLM sections stained with PAS-Richardson’s stain (D through F), pools of glycogen appear light purple and are well-preserved within hepatocytes. The hepatic architecture appeared normal at 4 months (Fig 3A,D), mildly altered in local areas at 12 months (Fig 3 B,E) and very distorted at age 16 months with increased fibrous areas (Fig 3 C,F). Low power view of trichrome stained paraffin sections illustrates the progressive hepatic fibrosis in canine GSD IIIa (Fig 4A, B, C), from periportal fibrosis, to bridging fibrosis and to cirrhosis at 4, 12 and 16 months, respectively. The evolution of these pathological processes was highlighted at higher magnification (Fig 4 D, E, F).
4. Progressive muscle damage caused by gradually increased glycogen accumulation

Progressive glycogen accumulation and tissue damage with age was detected in skeletal muscle of GSD IIIa dogs (Fig 5). At age 4 months, only low levels of glycogen accumulated within the cytoplasm of myocytes (Fig 5A), which is consistent with the observation that symptomatic myopathy is not commonly seen in young patients (Kishnani et al., 2010). All muscle cells were similarly affected. Under EM, the glycogen granules dispersed among the myofibrils and small blebs of sarcolemma containing glycogen deposits were readily seen beneath the cytoplasmic membrane (Fig 5D). At 12 months, the cytoplasmic glycogen began to pool around the periphery of the myotubes. Most cells were involved by glycogen accumulation, with a range from mild to severe involvement (Fig 5B). The large glycogen pools disrupted the contractile apparatus and caused fraying of myofibrils (Fig 5E). At 16 months, regions of the sarcoplasm were entirely occupied by cytoplasmic glycogen, displacing the contractile elements (Fig 5F). The histological findings matched the pattern of gradually increasing glycogen content measured in the muscle tissues (Fig 2B), and were also in concert with the trend of serum CPK activity (Fig 1D).

5. Glycogen deposition in adipocytes in muscle tissues

Fig 6 demonstrates the glycogen accumulation in adipocytes present in a muscle biopsy from one of the GSD IIIa dogs at 16 months of age. The appearance of PAS positive substances in adipocytes was an isolated event in our study, but it drew our attention to a potential disturbance of glycogen metabolism in adipocytes in GSD IIIa.
DISCUSSION:
GSD III is one of the most common glycogen storage diseases. Currently, disease progression and pathology are not well characterized. Other than symptomatic management, no therapy is available for this condition (Kishnani et al., 2010). There is an urgent need for an animal model to study disease progression and to develop effective therapies that are definitive or targeted and relevant to human treatment modalities. In the past decade, canine models have emerged as a powerful tool for studying hereditary diseases and for the development of new therapeutic approaches. For example, a canine model of GSD I had been established and successfully used for studying disease pathophysiology, long-term complications, and development of gene therapy (Kishnani et al., 2001; Koeberl et al., 2008). The naturally occurring GDE frame-shift mutation in CCR was first identified in 2007 (Gregory et al., 2007). The initial study of two affected dogs confirmed glycogen accumulation in liver and muscle and both dogs showed similar clinical signs to those of human disease (Gregory et al., 2007). A breeding colony was established to get a larger cohort of affected dogs with aims of understanding pathophysiological disease progression and developing novel therapies. The current study was designed to investigate in detail the natural history of the disease in this canine model.

Hypoglycemia and hyperlipidemia are dominant features in patients with GSD III in infancy and childhood (Hershkovitz et al., 1999; Geberhiwot et al., 2007; Bernier et al., 2008; Kishnani et al., 2010). Hyperlipidemia in human patients is possibly caused by increased lipid flux from adipose tissue to the liver as an alternative source of fuel in the setting of hypoglycemia (Bernier et al., 2008). However, none of the 8 affected dogs ever displayed signs of hypoglycemia during a 12-hour fasting, which could explain the normal concentrations of triglycerides and cholesterol.
throughout the study. In addition, there is a great difference in lipoprotein profiles and lipids metabolism between human and dog (Xenoulis and Steiner, 2010).

Liver and muscle damages are common features in GSD IIIa patients, and serum enzyme activities related to these organs are often elevated in the patients. Though abnormalities in serum enzyme activities have been repeatedly reported in previous clinical studies on GSD IIIa (Lucchiari et al., 2007; Karwowski et al., 2011), detailed correlation between enzyme levels and the states of disease progression has not been established. In this study of 8 affected dogs, we showed that measurements of both ALT and AST activity were elevated at a young age and continually increased throughout the experiment, indicating progressive liver damage. Though elevations of the two transaminases both indicate liver impairment, ALT is a more direct indicator as it is predominantly found in liver, while AST also broadly exists in other tissues, especially muscle (Goessling and Friedman, 2005). The steeper increase of the two enzymes after 12 months of age coincided with the increased liver fibrosis/cirrhosis as confirmed by histology, though increased AST could have also come from damaged muscle where this enzyme exists in significant amount (Weibrecht et al., 2010). High levels of ALP were noted in the dogs at different ages. Humans ALP exists in several isoforms and various conditions can lead to elevated serum ALP activity, but very high ALP activities are often of liver origin caused by severe intrahepatic cholestasis or bile duct obstruction (Sapey et al., 2000). Consistently elevated serum ALP activity in the GSD IIIa dogs appears primarily a result of hepatocyte swelling due to cytoplasmic glycogen accumulation, and had little correlation with the extent of fibrosis. CPK catalyzes the conversion of creatine to phosphocreatine, an energy reservoir for the rapid regeneration of ATP through the reverse reaction, in muscle and some neuronal tissues (Wallimann et al., 1992). High serum CPK activity is usually caused by injury or stress to the
muscle or heart tissue (Arts et al., 2007). The accelerated increase of CPK after 12 months, correlating with increased muscle glycogen content and damage, implied disruption of myocyte integrity that mimic the onset of phenotypic myopathy in adult patients. These parameters are useful in clinical diagnosis and evaluation of disease status, but whether they can be used as disease biomarkers needs to be further evaluated in this animal model.

High levels of glycogen were detected in liver at 4 months of age in all dogs. Liver glycogen content increased at 12 month followed by a significant decrease at 16 months. Progressive liver fibrosis was observed in this dog model. Liver fibrosis was minor at 4 months of age but extensive micronodular and macronodular cirrhosis was found in all 3 dogs analyzed at 16 months. The decline in glycogen content in liver from age 12 to 16 months was likely due to large scale replacement of hepatocytes by fibrous tissue, and correlated well with the observed accelerated increase of serum ALT and AST activities. Muscle glycogen content gradually increased with age. Disruption of the contractile apparatus and fraying of myofibrils were directly observed at 12 and 16 months as a result of large cytoplasmic glycogen deposition and correlated with sharply increased serum CPK and AST activities.

In addition to skeletal muscle, varied cardiac muscle involvement has been reported in patients with GSD IIIa. Ventricular hypertrophy is a frequent finding, but overt cardiac dysfunction or symptomatic cardiomyopathy is rare (Moses et al., 1989; Labrune et al., 1991; Hobson-Webb et al., 2010). A recent study demonstrated that a high-protein diet dramatically decreased the left ventricular mass index and serum creatine kinase levels and reversed cardiomyopathy in a patient with GSD IIIa (Dagli et al., 2009), indicating this treatment is a beneficial therapeutic choice for GSD IIIa patients with cardiomyopathy. Since it is not practical to perform frequent
myocardial biopsies on the dogs, cardiac muscle involvement was not a focus in this study. However, we did perform a less invasive electrophysiology (EP) study to test the electrical conduction system of the heart in four affected dogs at age 7 to 8 months, using a single catheter situated within the heart through femoral vein. Of the four dogs studied, two had atrial fibrillation upon electrical stimulation but all other conduction system characteristics were normal. The other two dogs were both within normal limits. Thus, at this time, there is no inclusive conclusion of cardiac involvement in this model.

It is interesting to find significant glycogen accumulation in adipocytes in an affected dog. Adipose tissue is a primary site for lipid storage. Glycogen is also found in adipocytes at a much lower concentrations than in liver and muscle, though its exact role remains unclear (Jurczak et al., 2007; Markan et al., 2010). Early studies suggested that glycogen is converted into fat in the adipose tissue and the dynamic regulation of adipose glycogen may serve as an energy sensor in coordinating glucose and lipid metabolism during the fasted to fed transition (Markan et al., 2010). In normal and nutritional balanced state, glycogen is histologically invisible in adipose tissue in rats (Fawcett, 1948). However, when rats are fed on a carbohydrate-rich diet after prolonged starvation, adipose glycogen was markedly increased (Tuerkischer and Wertheimer, 1942). In addition, administration of insulin in rats or dogs can result in a transient glycogen accumulation in the adipocytes (Fawcett, 1948). The ability to accumulate glycogen suggests that the synthesis of glycogen in adipocytes is also a dynamic process in GSD IIIa dogs. The clearance of adipocyte glycogen seems to be impaired by the lack of GDE activity in the affected dogs, suggesting that glycogen catabolism is through a mechanism similar to that in muscle and liver. To our knowledge, glycogen accumulation in adipose tissues has not been reported in patients with GSD IIIa and other GSDs, though one of us (PSK) has palpated a lipoma-like
structure in a GSD III individual. The existence of significant glycogen accumulation in adipocytes is unusual; whether the involvement of adipose tissue in GSD IIIa dogs is a species-specific event or a common feature needs to be further studied.

In this study, we demonstrated that the naturally occurring GSD IIIa dog model in CCR has a phenotype that closely resembles human disease, with glycogen accumulation in liver and skeletal muscle that lead to progressive hepatic fibrosis and myopathy. This disease model will help us better understand GSD IIIa disease progression, identify new biomarkers for the disease, and develop effective therapies such as enzyme replacement therapy, gene therapy, and substrate reduction therapy.

METHODS

Animals

The CCR breeding colony was maintained at Michigan State University. Housing, mating, rearing of offspring, and transport of dogs were performed in accordance with protocols approved by the Institutional Animal Care & Use Committee at MSU. Diagnosis of GSD IIIa was confirmed by mutation analysis (Gregory et al., 2007). A total of eight affected dogs of ages ranging from 2 to 6 months were transported to Duke University Medical Center for this study. The animals were reared on a normal canine diet at the Division of Laboratory Animal Resources (DLAR) of Duke University. All animal experiments were approved by the Institutional Animal Care & Use Committee at Duke University and were in accordance with the National Institutes of Health guidelines. All dogs were on regular diet that contains approximately 25 to 30% in protein and 45% in carbohydrate, respectively, throughout the study. For the first year of life, the dogs were fed with Science Diet puppy large breed dry food (Hill's
Pet Nutrition, Inc. Topeka, KS) by ad-libitum feeding for a few months and then getting 2.25 cups twice a day (4.5 cups/day) along with one can of puppy canned food (Science Diet® Puppy Gourmet Chicken Entrée) per day. After 1 year old all dogs were switched to normal adult food (Purina Lab Canine Diet 5006, Purina Mills, St. Louis, MO). Each dog got 3 cups of the dry food twice a day (6 cups/day) and also one can of Purina ProPlan Chicken & Rice Entrée (Nestlé Purina PetCare Company, St Louis, MO) wet food per day.

**Routine laboratory testing and tissue biopsy**

Blood (5 mL) was collected from saphenous or jugular veins for each dog every 4 weeks. Samples were sent to a commercial laboratory for a panel of routine biochemical tests, which included ALT, AST, ALP, CPK, glucose, triglycerides, cholesterol, bilirubin, albumin, urea nitrogen, gamma-glutamyl transpeptidase, and creatinine. Liver biopsies by laparotomy and skeletal muscle (quadriceps) biopsies were performed on each dog at specified ages under general anesthesia. Fresh tissue specimens were immediately frozen on dry ice and stored at -80°C until used for biochemical analysis, or placed in 3% glutaraldehyde or 10% neutral buffered formalin (NBF) for histology. All dogs were fasted but offered water for 12 hours prior to the surgical procedures.

**Tissue glycogen analysis**

Tissue glycogen content was assayed enzymatically using a protocol modified from Kikuchi et al (Kikuchi et al., 1998). Frozen liver or muscle tissues (50-100 mg) were homogenized in ice-cold de-ionized water (20 mL water/g tissue) and sonicated three times for 20 seconds with 30 seconds intervals between pulses, using a Misonix XL2020 ultrasonicator. Homogenates were clarified by centrifugation at 12,000 g for 20 minutes at 4 °C. Twenty µl of supernatant was
mixed with 55 µl of water, boiled for 3 minutes, and cooled to room temperature. Twenty five µl of amyloglucosidase (Sigma, cat log # A1602) solution (1:50 diluted into 0.1 M potassium acetate buffer, pH 5.5) was added, and the reaction was incubated at 37 °C for 90 minutes. Samples were boiled for 3 minutes to stop the reaction and centrifuged at top speed for 3 minutes in a bench-top microcentrifuge. Thirty µl of the supernatant was mixed with 1 mL of Infinity Glucose reagent (Thermo Scientific) and left at room temperature for at least 10 minutes. Absorbance at 340 nm was measured using a Shimadzu UV-1700 spectrophotometer. A reaction without amyloglucosidase was used for background correction for each sample. A standard curve was generated using standard glucose solutions in the reaction with Infinity Glucose reagent (0-120 µM final glucose concentration in reaction).

Histopathology

Fresh tissues were cut into 1-mm cubes and immersion-fixed overnight in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4 (Electron Microscopy Sciences, Hatfield, PA). The fixed tissues were then processed into Epon resin for high resolution light microscopy (HRLM) (Lynch et al., 2005). Tissue sections were cut at 1 µm, and stained with periodic acid–Schiff (PAS) -Richardson’s stain for glycogen observation. Additional ultrathin sections (70 nm) were cut, stained and examined by electron microscopy (EM). In addition, small pieces of fresh tissues were fixed in 10% NBF and processed into paraffin blocks, cut into 5 µm sections and stained with hematoxylin and eosin (H&E) or trichrome stains.

Statistical analysis of glycogen content

The significance of differences between two different time points was assessed using two-tailed, unequal variance student T-test. A p-value <0.05 was considered to be statistically significant.
ACKNOWLEDGEMENTS

We wish to acknowledge inspiration and support from the Workman family of Lowell, Indiana. We deeply appreciate assistance from the Duke DLAR staff in animal care and procedures. We wish to acknowledge excellent technical support from Elizabeth Drake, Keri Fredrickson, and Stuti Das, and expertise advice from Dr. Y.T. Chen.

FUNDING

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

COMPETING INTERESTS

The authors declare that they do not have any competing or financial interests.

AUTHOR CONTRIBUTIONS

H.Y. and B.S. performed the major experiments and wrote the manuscript; B.L.T performed histology of tissue biopsies; S.C. performed surgical procedures; J.F. maintained and genotyped the CCR colony; P.S.K., D.D. K., and S.A. provided clinical advice and technical support. B.S. and P.S.K. designed the experiments and supervised the study.

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**TRANSLATIONAL IMPACT BOX**

Liver and muscle are major affected tissues in human patients with GSD IIIa, a hereditary disease caused by mutations in the glycogen debranching enzyme (GDE) gene. Liver symptoms often appear in childhood and typically improve after puberty in most patients, but liver cirrhosis and hepatic adenoma or hepatocellular carcinoma have been reported in some cases. Progressive myopathy and cardiomyopathy are a major cause of morbidity in adults. There is significant clinical variability in the severity of the myopathy and cardiomyopathy symptoms caused by different types of mutations scattered throughout the 85-kb glycogen debranching enzyme gene.

The natural history of GSD IIIa disease progression has not been well established in human patients and there is no effective treatment for this disease. The absence of an adequate animal model is another major obstacle to progress in understanding the mechanisms and pathogenesis of the disease and evaluating feasibility of a novel therapy. As the search for an effective
treatment for GSD IIIa is ongoing and the pathophysiology of the disease and mechanisms of
clinical variability are not well understood, an appropriate animal model that mimics human
disease is needed. Recently GSD IIIa was identified in curly-coated retrievers (CCR). The
affected dogs carry a frame-shift mutation predicting deletion of the C-terminal 126 amino acids
of GDE. The dogs showed no detectable GDE enzyme activity in liver and muscle, resulting in a
GSD IIIa phenotype. However a thorough characterization of this model has not been performed.

In this study, the authors characterized in detail the disease expression and progression in the
affected dogs. Abnormally high glycogen deposition was found in liver and muscle of these
dogs, along with elevated and gradually increasing serum enzyme (AST, ALT, ALP, and CPK)
activities. Progressive, age-related fibrosis was also seen in livers. In muscle, increasing
glycogen deposition was observed, accompanied by disruption of the contractile apparatus and
fraying of myofibrils. Progressive liver fibrosis and muscle damage caused by glycogen
accumulation are the major features of the CCR dogs. The affected dogs closely resembled
human patients and thus are an accurate model of GSD IIIa. As canine models are emerging as a
powerful tool for the study of hereditary diseases and development of new therapeutic
approaches, the findings in this research will greatly improve our understanding of the disease
progression and allow opportunities to develop disease biomarkers and investigate treatment
interventions in this animal model.

In future studies, the difference in disease progression between the muscle and liver is an
interesting aspect, as liver symptoms alleviate with age while muscle symptoms worsen. The
authors are also making efforts in evaluating various potential treatments such as high-protein
diet management, gene therapy, enzyme replacement therapy, and blockage of glycogen synthesis by existing drugs.
FIGURE LEGENDS

**Fig. 1.** Serum enzyme activities and lipid levels in GSD IIIa-affected CCRs. Blood was collected every month and routine serum biochemistry determinations were performed. ALT, alanine transaminase; AST, aspartate transaminase; ALP, alkaline phosphatase; CPK, creatine phosphokinase. Values were average of 3 to 6 dogs at each time point. Shaded areas indicate normal ranges.

**Fig. 2.** Fasting glycogen contents in liver and skeletal muscle of GSD IIIa-affected CCRs. Values of each time point were derived from 4 dogs at 4 and 12 months, and 3 dogs at 16 months, with 2 pieces of the same tissue for each dog. Average and standard deviation are shown. Ctrl, average of triplicate specimens from a 6-month-old normal dog.

**Fig. 3.** Marked glycogen accumulation is present in hepatocytes at 4, 12 and 16 months of age. Panels A through C. Paraffin-embedded, hematoxylin and eosin-stained liver sections illustrate the typical vacuolated appearance of glycogen filled hepatocytes at 4, 12 and 16 months. In high resolution light microscopy sections (D through F) stained with PAS-Richardsons stain, the glycogen is well-preserved and appears light purple. Dense fibrosis is evident in panel F; fibroblasts stain light blue. (Magnification of A, B and F: 400x, scale bar = 50 microns; magnification of C: 200x, scale bar = 100 microns; magnification of D and E: 600x, scale bar = 30 microns).

**Fig. 4.** Progressive hepatic fibrosis is a feature of canine GSD3. Panels A through C show the progression from periportal fibrosis, to bridging fibrosis to cirrhosis at 4, 12 and 16 months,
respectively (paraffin sections, trichrome stain, 20x; scale bar = 300 microns). Panels D through F show the evolution of these pathological processes at higher magnification (paraffin sections, trichrome stain, 100x; scale bar = 100 microns).

**Fig.5.** Progressive cytoplasmic glycogen accumulation occurs in skeletal muscle of GSD3 dogs. Panels A through C show the progressive accumulation of glycogen in skeletal muscle over time. MetaMorph measurements were 6.5±3.1, 20.3±7.6, and 17.3±4.7 percent tissue area occupied by glycogen at 4, 12 and 16 months, respectively. (high resolution light microscopy, PAS/Richardson’s stain, magnification 400x, scale bar = 50 microns). Panels D through F show the ultrastructural changes which occur over time. At 4 months, glycogen accumulates in the cytoplasm and dissects in between myofibrils and just beneath the cytoplasmic membrane, forming small blebs (panel D, magnification 6000x, scale bar = 2 microns). At 12 months, the cytoplasmic glycogen begins to pool and disrupts the contractile apparatus, causing fraying of myofibrils (panel E, magnification 2500x, scale bar = 5 microns). At 16 months, entire regions of cells are filled with glycogen, displacing all contractile elements, leaving only mitochondria to float within the pools of glycogen (panel F, magnification 2000x, scale bar = 6 microns).

**Fig. 6.** The accumulation of cytoplasmic glycogen within adipocytes is apparent in 16 month biopsies. Panel A: glycogen stains purple at the periphery of fat globules within adipocytes present in skeletal muscle biopsies (high resolution light microscopy, PAS/Richardson’s stain, magnification 600x, scale bar = 30 microns). Panel B: Electron microscopy demonstrated the finely granular ultrastructure of the glycogen surrounding fat globules in adipocytes (magnification 2500x, scale bar = 5 microns).
Fig 3

(A) 4 months

(B) 12 months

(C) 16 months

(D) (400x)

(E)

(F)
Fig 6