

## ABSTRACT

CRANE, BAYLEY A. Efficacy of Gene Therapy in Dogs with Glycogen Storage Disease Type Ia. (Under the direction of Drs. David Dorman and Malcolm Roberts).

Glycogen storage diseases (GSD) are inherited metabolic disorders that affect glycogen use and storage. People with GSD Ia lack the enzyme glucose-6-phosphatase (G6Pase). As a result, these people are unable to convert liver glycogen to free glucose and develop severe hypoglycemia. Patients with GSD also develop growth retardation, hepatomegaly, renomegaly, hypertriglyceridemia, hypercholesterolemia, and hyperlactacidemia. No cure for GSD Ia currently exists. Patients are treated symptomatically with repeated naso-gastric feedings and glucose infusions to maintain normal blood glucose concentrations. Despite treatment, the underlying enzymatic defect remains. Gene therapy holds the promise of correcting this metabolic defect, thus providing a true cure for GSD Ia. Gene therapy uses modified virus particles to deliver a replacement functional G6Pase gene to the patient's liver. Our group is using two viral vectors, adeno-associated virus (AAV) and helper-dependent adenovirus (HDAd), for gene therapy in dogs with an inheritable form of GSD Ia. We have treated three GSD Ia dogs with the AAV vector and two GSD Ia dogs with the HDAd vector. Vector-treated dogs were able to maintain normal blood glucose concentrations and unlike their untreated counterparts, survived for several years. These promising results provide hope that gene therapy may emerge as an effective treatment for people with GSD Ia.

Efficacy of Gene Therapy in Dogs with Glycogen Storage Disease Type Ia

by  
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I was born on October 24, 1982 in Fayetteville, North Carolina to Edward and Ann Crane. I attended South View Senior High School and graduated in June 2001. I received my B.S. in Biology at the University of North Carolina at Chapel Hill and graduated in May 2005. Upon graduation, I worked for Dr. Susan Tonkonogy at North Carolina State University – College of Veterinary Medicine as a laboratory technician. My involvement in activities within the CVM led me to pursue a Master of Science degree in Physiology. I will complete my M.S. in Physiology at North Carolina State and graduate in May 2009.

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## INTRODUCTION

### Glucose Homeostasis

Glucose homeostasis plays a critical role in maintaining euglycemia within the body. In mammals, postprandial hyperglycemia stimulates the production of the hormone insulin within pancreatic  $\beta$ -cells. Insulin release promotes the uptake and storage of glucose by the body's tissues and conversion to glycogen. During hypoglycemia, the hormone glucagon is secreted from pancreatic  $\alpha$ -cells. The release of glucagon prompts the liver to convert the stored glycogen to glucose.

Glucose homeostasis is primarily regulated by glycogenesis and glycogenolysis (Pilkis and Granner, 1992). Glycogenesis is a multi-component process that converts free glucose into glycogen. Glucose enters the liver via passive transport down the glucose concentration gradient, mediated by a specialized glucose transport protein, GLUT2 (Roach, 2002). Glucose is then converted to glucose-6-phosphate (G6P) via glucokinase. G6P is converted to glucose-1-phosphate (G1P) via the enzyme phosphoglucomutase (Chen, 2001). G1P molecules are converted into UDP-glucose by the action of uridyl transferase (UDPG-pyrophosphorylase) resulting in the production of glucose and pyrophosphate (Chen, 2001). The UDP-glucose molecules are synthesized together in a chain by glycogen synthase, which act on an existing glycogen primer or glycogenin, a small protein that forms the primer. The pyrophosphate is hydrolyzed by pyrophosphatase into two molecules of inorganic phosphate (Chen, 2001). Lastly, glycogen branching enzyme breaks the  $\alpha$ -1,4 glycosidic linkages from

the outer end of the chain, and results in the development of  $\alpha$ -1.6 glycosidic linkages, providing a more stable glycogen molecule that allows for more efficient storage and mobilization of glucose (Roach, 2002; Voet, 2002).

Glycogenolysis is the process by which glycogen is sequentially catabolized into G1P and G6P. Glycogenolysis is mediated by numerous enzymes. Glycogen phosphorylase catalyzes the breakdown of glycogen to G1P. Glycogen debranching enzyme is responsible for removing the branches of the glycogen molecule, thereby giving glycogen phosphorylase access to additional glucose residues. The enzyme phosphoglucomutase converts G1P to G6P. Lastly, glucose-6-phosphatase (G6Pase) converts the G6P molecule into free glucose which leaves the cell via a glucose transporter and is carried by the blood to surrounding tissues (Voet, 2002).

Alterations of the enzymes involved in glucose and glycogen metabolism can have adverse health outcomes. In particular, a family of glycogen storage diseases (GSD) emerge due to spontaneous mutations in these key metabolic enzymes.

### **Historical Background of Glycogen Storage Disease (GSD)**

GSDs represent a class of inherited metabolic disorders affecting glycogen metabolism (Chen, 2001). Most GSDs affect the liver and/or muscle as these are the tissues that store the most glycogen in the body. There are numerous types of GSD, all distinguishable by a deficient or missing protein (Table 1). GSDs are categorized by number

and in accordance with the order in which they were discovered (Chen, 2001). To date, there are 13 (including subtypes) reported GSDs.

The first clinical description of a GSD occurred in 1929 by von Gierke in the landmark paper “Hepatonephromegalia glycogenica”. The disease recognized by von Gierke has subsequently been identified as GSD Type Ia. GSD Type Ia, or von Gierke Disease, is an autosomal recessive disorder caused by a deficiency of the G6Pase system. GSD Ia patients lack a functional G6Pase enzyme and have disrupted glucose homeostasis resulting in decreased hepatic glucose production, excess hepatic glycogen accumulation, and increased concentrations of hepatic G6P (Chen, 2001). Thus, alternative metabolic pathways play a major role in regulating glucose concentrations within the body. Due to the liver’s inability to convert glycogen to free glucose, this excess glycogen is converted to G6P. G6P undergoes glycolysis, where it is broken down into pyruvate to yield ATP as an energy source.

GSD Type II is commonly referred to as Pompe Disease. This disease progressively affects skeletal muscle (Chen, 2001). GSD Type II is an autosomal recessive lysosomal storage disease caused by a deficiency of acid  $\alpha$ -glucosidase, the enzyme that degrades the glycosidic linkages between glucose residues (Raben et al., 2002). This disease results in an accumulation of glycogen within lysosomes, disrupting the normal function of the cell and eventually leading to muscle fatigue, muscle weakness, and tissue destruction (Raben et al., 2002; Fernandes and Smit, 2000).

A deficiency in the glycogen debranching enzyme causes Type III GSD. This disease is also referred to as Cori Disease or Forbes Disease. GSD Type III is an autosomal

recessive disorder that results in accumulation of glycogen in the liver and muscle due to the inability to debranch glycogen (Shen and Chen, 2002). A deficiency in this enzyme causes an inability to break down glycogen which results in the formation of an abnormal glycogen structure, which cannot be converted into free glucose.

GSD Type IV, also referred to as Andersen disease, is caused by a deficiency of the glycogen branching enzyme (Moses and Parvari, 2002). GSD Type IV is a rare autosomal recessive disorder in which the deficient enzyme causes insufficient branching of the glycogen molecule, giving it a structure similar to amylopectin (Fernandes and Smit, 2000; Ozen, 2007). The abnormally structured glycogen accumulates in the liver and muscle and is thought to trigger an inflammatory reaction in which the body begins attacking the glycogen and the tissues in which it is stored (Chen, 2001). The end result is cirrhosis of the liver and other storage tissues.

McArdle Disease, or Type V GSD, is an autosomal recessive disorder caused by reduced muscle phosphorylase activity, the enzyme that plays a critical role in the conversion of glycogen to glucose during anaerobic exercise (Chen, 2001). A deficiency in this enzyme prevents glycogen catabolism and results in glycogen accumulation in muscle tissues. To generate energy muscle is degraded, which results in muscle fatigue, pain, and cramping (Chen, 2001). This disease is one of the most common metabolic disorders causing exercise intolerance and recurrent myoglobinuria in people (DiMauro et al., 2002).

Type VI GSD, or Hers disease, is a rare X-linked disease, resulting from a deficiency in liver phosphorylase (Chen, 2001). This enzyme catalyzes glycogen degradation by releasing glucose-1-phosphate (G1P) from the  $\alpha$ -1,4-glycosidic bond (Roach, 2002). GSD

Type VI closely resembles GSD Type I, as a deficiency in the liver phosphorylase prohibits glycogen from being broken down into free glucose. Unlike GSD Type I, GSD Type VI represents a very mild and manageable form of GSD because gluconeogenesis is still able to produce glucose from non-carbohydrate sources (Chen, 2001).

A deficiency in muscle phosphofructokinase forms the basis for GSD Type VII, or Tauri disease. GSD Type VII is an autosomal recessive disorder, impairing the conversion of fructose-6-phosphate (F6P) to fructose 1,6 biphosphate (F1,6BP), a rate limiting step in glycolysis (Nakajima et al., 2002). The clinical features of this disease closely resemble those of GSD Type V, but GSD Type VII is much more severe due in part to an accumulation of an abnormal form of glycogen, resembling amylopectin, in muscle fibers (Chen, 2001).

Individuals with GSD Type IX have a deficiency in phosphorylase kinase (Fernandes and Smit, 2000). Phosphorylase kinase is a regulatory enzyme that aids in the breakdown of glycogen in response to neural or hormonal stimulation (Chen, 2001). GSD Type IX, can be autosomal recessive, or X-linked and leads to excess glycogen accumulation in the liver or muscle. A deficiency in the phosphorylase kinase activity of the liver represents a disease similar to GSD Type VI (Chen, 2001).

GSD Type XI, also known as Faconi-Bickel Syndrome, is a rare autosomal recessive disorder caused by a mutation in the GLUT2 gene, an important facilitative glucose transporter in hepatocytes, pancreatic  $\beta$  cells, enterocytes, and renal tubular cells (Santer et al., 2002). This defect impairs the transport of monosaccharides, such as glucose, across

cellular membranes (Ozen, 2007). A defect in glucose transport causes glycogen accumulation in the liver and kidneys and renal tubular dysfunction (Chen, 2001).

Lastly, GSD Type 0 represents a deficiency in the enzyme glycogen synthase. Glycogen synthase catalyzes the synthesis of glycogen by transferring glucose units from UDP-glucose to a glycogen primer (Chen, 2001). Symptoms seen with GSD Type 0 resemble that of GSD Type I, however, hepatomegaly is not present (Ozen, 2007). GSD Type 0 represents a rare autosomal recessive disorder where the enzyme defect leads to hypoglycogenesis instead of glycogen storage, because glycogen synthesis is reduced (Fernandes and Smit, 2000). Even though this disease does not result in glycogen storage, it is still considered a GSD because it shares some of the same metabolic characteristics as the other GSDs (Ozen, 2007).

### **History of GSD Ia**

GSD Ia was first observed in 1929 when an 8-year-old girl presented with hepatomegaly. Described as “hepatonephromegalia glycogenica”, von Gierke recorded the first clinical, pathological, microscopic, and chemical observations of abnormal liver glycogen storage (von Gierke, 1929; Moses, 2002).

Cori and Cori (1952) determined that the primary cause of GSD Type Ia was a G6Pase deficiency in hepatic tissues. They found that patients lacking this enzyme had excess glycogen accumulation in the liver and a tendency to become hypoglycemic.

Historically, this was the first time an enzyme deficiency had been proven to result in a clinical disorder (Chou et al., 2002a).

Senior and Loridan (1968) identified two subtypes of GSD Ia. The first subclass represented patients having no G6Pase activity and was classified as GSD Type Ia. The second subclass includes patients with reduced G6Pase activity and was denoted as GSD Type 1b (Senior and Loridan, 1968).

Arion et al. (1980) established that the conversion of G6P to glucose involves several membrane proteins. These proteins were identified as a G6Pase catalytic subunit, a G6P transporter, a phosphate transporter, and a glucose transporter (Chou et al., 2002a). This multi-component enzyme system is essential in maintaining glucose homeostasis. This discovery led to the identification and classification of four different subtypes in Type I GSDs. The subtypes were characterized by a mutation within the following enzyme/protein: 1) G6Pase catalytic subunit (GSD-1a); 2) G6P transporter, G6PT (GSD-1b); 3) phosphate transporter, T2 (GSD-1c); and 4) glucose transporter, T3 (GSD-1d) (Chou et al., 2002a; Chen, 2001).

Most recently, it was hypothesized that only two subtypes of Type I GSD exist, GSD Ia and GSD non-Ia. Chen et al. (2008) identified clinical cases of GSD Type Ic, proposed to be deficient in the phosphate transporter, and found that each patient had a deleterious mutation in the G6PT gene, suggesting that the G6PT is both the G6P and phosphate transporter. In fact, GSD Type Ic has not been characterized at the molecular level, and the existence of GSD Id remains unproven (Chen et al., 2008). Therefore, GSD Type I subtypes are now identified as Type Ia and Type non-Ia.

## Glucose-6-Phosphatase

The biochemical and molecular basis of GSD Type Ia relates to a deficiency in the enzyme glucose-6-phosphatase (G6Pase). This enzyme is responsible for forming significant amounts of glucose in the liver for circulation throughout the body (Chen, 2001).

G6Pase is one part of a multi-component “G6Pase system” found primarily in the liver. G6Pase is a nine-transmembrane, helical protein found on the membrane of the endoplasmic reticulum (ER) that catalyzes the hydrolysis of G6P to free glucose in the final pathways of glycogenolysis and gluconeogenesis (Shieh et al., 2002; Van Schaftingen and Gerin, 2002). The active site of the enzyme is oriented toward the lumen of the ER, rather than exposed to the cytoplasm as previously predicted (Arion et al., 1980). The catalytic subunit of the G6Pase is a transmembrane phosphohydrolase and the rate limiting step in the enzymatic reaction is the transport of the substrate (G6P) across the microsomal membrane (Janecke et al. 2001). The primary amino acid residues that comprise the catalytic center of G6Pase are Lys<sup>76</sup>, Arg<sup>83</sup>, His<sup>119</sup>, Arg<sup>170</sup>, and His<sup>176</sup>. It is predicted that a mutation in any of these residues would inactivate the enzyme (Ghosh et al., 2002).

Another important component of the G6Pase system is the microsomal glucose-6-phosphate transporter (G6PT) (Senior and Loridan, 1968). This transporter aids in the transport of G6P across the ER membrane. A phosphate transporter (T2) was also identified, aiding in the transport of the phosphate derived from hydrolysis out of the ER. However, recent studies suggest that the G6PT is an antiporter, transporting G6P into the cell and the phosphate from G6P hydrolysis out of the cell (Chen et al., 2008). Patients that were



originally thought to have GSD Ic, were shown to have a deleterious mutation in the G6PT protein. Therefore, a deficiency in the G6PT protein is now thought to result in GSD Type non-Ia.

The final component in the G6Pase system is the microsomal glucose transporter (T3). Upon hydrolysis of G6P, this protein is responsible for the transport of free glucose from the lumen of the ER to the blood (Chou and Mansfield, 1999). It was originally suggested that a deficiency in the T3 protein results in GSD Type Id, but this remains unproven and currently evidence for this theory does not exist (Chen et al., 2008).

Molecular characterization of the G6Pase enzyme was initially difficult because of its hydrophobicity and tight association with the ER membrane (Chou and Mansfield, 1999). Shelly et al. (1993) successfully isolated a murine G6Pase cDNA library by screening a mouse liver. The human G6Pase cDNA was isolated as a result of its homology with the murine cDNA (Lei et al., 1994). Kishnani et al. (1997) successfully isolated canine cDNA G6Pase from two Maltese puppies.

The structural organization of the murine, human, and canine G6Pase genes has been determined. All genes are 10-12 kb single copy genes composed of five exons (Chou and Mansfield, 1999). The human G6Pase gene is located on chromosome 17g21 (Chen, 2001). All three mammalian G6Pases consist of 357 amino acids residues, sharing 90% homology with each other (Chou et al., 2002a).

There are 76 separate G6Pase mutations that have been found in over 400 GSD Ia patients (Chou et al., 2002a). Most of these mutations are missense mutations, but nonsense, deletion, and insertion mutations have also been identified (Chou et al., 2002a). The

missense mutations have been grouped into three categories: active site, helical, and non-helical mutations, based on their location within the G6Pase gene (Shieh et al., 2002). Mutations within the G6Pase active site comprised of amino acid residues Lys<sup>76</sup>, Arg<sup>83</sup>, His<sup>119</sup>, Arg<sup>170</sup>, and His<sup>176</sup>, completely abolish the enzyme's activity. Helical and non-helical mutations can either abolish or greatly reduce G6Pase activity (Shieh et al., 2002). There may be an association of some mutations with more or less severe phenotypes (Chou et al., 2002a).

### **Clinical Manifestations in Patients with GSD Ia**

Patients with GSD Ia are unable to break down stored glycogen into free glucose, resulting in excessive hepatic glycogen accumulation. Consequently, one of the major clinical manifestations of GSD Ia is hepatomegaly. The liver of a GSD Ia patient can represent 20% of that patient's body weight (Chou et al, 2002a). The enlarged liver may be present at birth or may become detectable within the first few months of life (Chen, 2001).

Another hallmark of GSD is hypoglycemia, usually occurring after a fast, as short as 20-30 minutes (Chen, 2001). Hypoglycemia occurs once exogenous sources of glucose are exhausted. The body corrects hypoglycemia and generates glucose by catabolizing glycogen stores via glycogenolysis or synthesizing glucose from non-carbohydrate sources via gluconeogenesis. However, in patients with GSD Ia, the deficiency of the G6Pase enzyme impairs the final steps in glycogenolytic and gluconeogenic activity, inhibiting the production of free glucose (Chen, 2001; Fernandes and Smit, 2000).

Patients with GSD Ia exhibit moderate to severe growth retardation, a result of a deficiency in the body's metabolic processes, doll-like faces, and a short stature with relatively thin extremities (Chen, 2001).

Hyperlipidemia, or increased lipid concentrations in the blood, occurs in patients with GSD Ia. Hyperlipidemia is associated with increased plasma concentrations of cholesterol and/or triglycerides (Nelson et al., 1999). The major contributing factor to GSD-induced hyperlipidemia is increased lipolysis due to decreased hepatic glucose production (Chou et al., 2002a; Chen, 2001). This increased glycolytic activity and the subsequent production of lactate and pyruvate, results in the production of substrates (acetyl-CoA, glycerol) and cofactors (NADH, NADPH) which ultimately leads to increased lipid synthesis (Chou et al., 2002a). Increased lipid synthesis results in fatty deposits, primarily in the liver, which contributes to hepatomegaly. Due to the elevation of triglycerides, plasma of patients with GSD often has a milky appearance (Chen, 2001).

Patients with GSD Ia present with lactic acidosis during the neonatal period, continuing into adulthood (Chen, 2001). In a normal patient, glycogenolysis is the primary pathway providing the body with free glucose. In glycogenolysis, glycogen is converted into G1P, which is converted to free glucose by G6Pase. In patients with GSD Ia, a deficiency in the G6Pase enzyme does not permit this conversion of glycogen to free glucose. Instead, the glycolytic pathway predominates and the resulting excess G6P is ultimately converted into pyruvate. During anaerobic metabolism, pyruvate is metabolized into lactate. Increased lactate levels can provide the body with glucose through gluconeogenesis in the liver;

however lactic acidosis results when blood lactate levels are too high (Chen, 2001; Fernandes and Smit, 2000). Hyperlactacidemia can be fatal.

## **GSD Therapies**

Currently, there is no cure for GSD Ia and this disease can be fatal if not treated (Chen, 2001; Chou et al., 2002b). Historically, management of hypoglycemia with dietary therapy and frequent feedings has been a standard therapy for patients with GSD Ia (Williams, 1986). Dietary treatment for humans with GSD Ia involves frequent feedings, oral administration of uncooked cornstarch (a slow-release form of glucose), and continuous night-time feeding via nasogastric tube (Chen, 2001). Proper dietary management plays a pivotal role in minimizing the metabolic abnormalities and long-term complications associated with the disease.

While dietary therapy is an effective means of maintaining euglycemia, it does not correct the underlying pathological complications (Chou and Mansfield, 2007). Even with dietary therapy, patients with GSD Ia continue to suffer from biochemical abnormalities such as hyperlipidemia and hyperlactacidemia (Chen, 2001). These recurring metabolic abnormalities persist through adulthood and can result in long-term complications including, short stature, renal disease, and hepatic adenomas (Chou and Mansfield, 2007).

The effectiveness of dietary therapy is influenced by patient compliance. Continuous nasogastric infusion requires frequent monitoring to insure that the equipment does not malfunction and the patient is receiving required nutrients (Chen et al., 1984). If patients do

not comply with the dietary methods, normoglycemia cannot be maintained. The inability to correct biochemical abnormalities and the lack of patient compliance, suggest the need to explore other treatment options for GSD Ia.

Another therapy offered to patients afflicted with GSD Ia is liver transplant. This represents a type of curative therapy that could correct the biochemical abnormalities associated with the disease (Chen, 2001). Though liver transplantation offers a promising therapy for patients afflicted with GSD Ia, there are many associated limitations. First, liver transplantations are extremely expensive. This factor alone limits the percentage of GSD Ia patients that could have access to this therapy. Liver transplants are highly invasive. As donor organs are scarce this therapeutic option is unavailable to the majority of patients afflicted with GSD Ia (Matern et al., 1999). Lastly, the primary criteria for liver transplantation are usually enlarging adenomas and/or concern about hepatocellular carcinoma (HCC). There is limited evidence of the value of liver transplants as effective long-term therapeutic methods, but some suggest that renal disease may be reversed or corrected (Matern et al., 1999; Chen, 2001; Chou and Mansfield, 2007). Consequently, alternative treatment strategies are required.

Gene therapy represents an alternative therapeutic approach for patients with GSD Ia. Gene therapy consists of using a vector to deliver a therapeutic gene to a target cell. The most common type of vector is a virus, which encapsulates, replicates, and delivers the gene of interest in a pathogenic manner by invading target cells and replicating. The ultimate goal of gene therapy is to attain stable, long-term transgene expression in the target tissue.

The adenovirus (Ad) is a non-enveloped, double-stranded DNA virus (Gardlik et al., 2005). There are more than 50 serotypes of adenoviruses, but serotypes 2 and 5 are most commonly used in gene therapy (Gardlik et al., 2005). The use of Ad vectors in gene therapy has been limited due to the toxic nature of the virus and its short duration of transgene expression (Zingone et al., 2000). As the majority of the human population has been exposed to Ad infection (i.e. the common cold) and has antibodies against different Ad serotypes, 90% of injected Ad vectors are degraded within 24 hours (Gardlik et al., 2005). This increased immune response leads to short-term expression of the transgene and an increase in pro-inflammatory cytokines and infiltration of leukocytes and neutrophils into transduced tissues (Zaiss et al., 2002; Jozkowicz and Dulak, 2005). The short-term expression and heightened immune response illustrate why the use of Ad vector-mediated therapies has been limited and why alternative vectors have been considered.

To achieve long term expression of the gene of interest, a more stable vector is required. The adeno-associated virus (AAV) is a single-stranded DNA virus belonging to the parvovirus family (Gardlik et al., 2005). AAV vectors are described as being relatively stable, highly efficacious, and having broad tissue tropism (Gardlik et al., 2005). AAV gene therapy has successfully been used in human phase I clinical trials for the treatment of hemophilia B (Snyder and Francis, 2005). The most extensively studied and commonly used AAV vector is the AAV vector, serotype 2, although other serotypes are used (Gardlik et al., 2005). The AAV2 serotype is used most frequently because it has a natural tropism for specific tissues, such as muscle and liver following intravenous administration (Beaty et al.,

2002). These characteristics make the AAV vector a promising candidate for GSD gene therapy.

Another promising candidate for gene therapy is a helper-dependent adenovirus (HDAd). This is an Ad vector in which all viral coding sequences have been removed, leaving only sequences for packaging and vector propagation (Jozkowicz and Dulak, 2005). Compared to Ad or AAV vectors, the lack of viral particles in the HDAd vector, suggests that this vehicle would be less immunogenic and result in stronger transgene expression. HDAd vectors preferentially transport the gene of interest to the liver, where infection efficacy may reach almost 100% (Jozkowicz and Dulak, 2005). It has been shown in baboons and mice that a single HDAd delivery can result in strong transgene expression, lasting 1 to 2.5 years (Morrall et al., 1999; Jozkowicz and Dulak, 2005). Due to the long-term transgene expression and relative low toxicity, HDAd viral vectors offer a promising tool for gene therapy.

### **GSD Animal Models**

The use of animal models to study human disease strongly depends on the homology of the animal and human conditions and the overall genetic similarity among species (Walvoort, 1983). Animal disease models of GSD Ia include the G6Pase knock-out (KO) mouse model and the dog model of GSD Ia. These models mimic the GSD Ia human disorder, with the exception of hyperlactacidemia in mice. Affected animals have hypoglycemia, hyperlipidemia, excess glycogen accumulation, decreased hepatic G6Pase

concentrations, and exhibit growth retardation. Dogs affected with GSD Ia present with similar clinical abnormalities as children with GSD Ia, such as growth retardation and hepatomegaly (Figure 1). The reported 90% amino acid sequence homology between the murine, human, and canine genetic sequence supports the use of these models as extremely valuable tools in studying GSD Ia. (NOTE - For the purpose of this thesis the following terms are used; G6Pase<sup>-/-</sup> mouse (has GSD Ia disease), affected (homozygous recessive, has GSD Ia disease), carrier (heterozygous, but clinically unaffected), normal or wild type (non-carrier, clinically normal).

Lei and coworkers (1996) developed a G6Pase<sup>-/-</sup> mouse that mimicked the pathophysiology of the human GSD-Ia patients. These G6Pase<sup>-/-</sup> mice manifested the same phenotype as human GSD-Ia patients, presenting with clinical conditions such as hypoglycemia, hepatomegaly, growth retardation, and hyperlipidemia. G6Pase<sup>-/-</sup> mice exhibit hypoglycemic seizures and have lower blood glucose concentrations than their unaffected littermates (Lei et al., 1996; Zingone et al., 2000). In the absence of any dietary therapy, G6Pase<sup>-/-</sup> mice die within 2 weeks of life (Chou et al., 2002b). The G6Pase<sup>-/-</sup> mice weigh significantly less than the wild type mice and carrier mice, indicating significant growth retardation, plus they have increased glycogen accumulation and increased fat deposition in the liver (Lei et al., 1996). G6Pase<sup>-/-</sup> mice have no detectable G6Pase activity and Western blot analysis confirms the absence of G6Pase protein (Lei et al., 1996). However, the absence of lactic acidosis (a prominent clinical manifestation in human GSD Ia) in the G6Pase<sup>-/-</sup> mice is a limiting factor when using this model. In the mouse, the lactate



produced by glycolysis may be more readily consumed by the liver for fatty acid synthesis thus, limiting the development of lactic acidosis (Lei et al., 1996).

The first naturally occurring G6Pase mutation in animals was identified by Brix and coworkers (1996) when two 47-day-old littermate Maltese puppies presented with a history of failure to thrive, central nervous system (CNS) depression, and poor body condition. At necropsy, it was discovered that the puppies had severe growth retardation, distended abdomens, and grossly enlarged, pale livers (Brix et al., 1995). Histological analysis revealed excess glycogen accumulation in the liver while biochemical analysis showed that the puppies' hepatic G6Pase activity was significantly reduced (Brix et al., 1995). Due to limitations in size within the Maltese breed, a novel GSD Ia dog model was established by crossbreeding male Maltese dogs carrying the mutated, deficient G6Pase gene with Beagles (Kishnani et al., 2001). The crossbreeding resulted in increased litter size and greater numbers of affected puppies that had the typical clinical signs associated with GSD Ia in humans (Kishnani et al., 2001). Untreated puppies with GSD Ia died between 5 and 8 weeks of age. Much like humans with GSD Ia, affected Maltese-Beagle puppies exhibited hypoglycemia, growth retardation, hepatomegaly, hypercholesterolemia, hypertriglyceridemia, and hyperlactacidemia. Vacuolated hepatocytes due to glycogen and lipid accumulation were described (Kishnani et al., 2001). Hepatic G6Pase levels varied between 0.5 and 0.7 mmol/min/g tissue in two affected puppies, compared to approximately 8 mmol/min/g tissue in age-matched control puppies (Kishnani et al., 2001).

## Gene Therapy in Animal Models of GSD

The development of these GSD Ia animal models, closely mimicking the human disorder, has been instrumental in the development of vector-mediated gene therapy as an alternative method of therapy for GSD Ia. Vector-mediated gene therapy in G6Pase<sup>-/-</sup> mice model and GSD Ia puppies has not only increased survival, but has also ameliorated many of the biochemical abnormalities associated with the disease.

Initially, a single-dose of an Ad vector carrying a murine-G6Pase transgene corrected hypoglycemia and other biochemical abnormalities in G6Pase<sup>-/-</sup> mice (Zingone et al., 2000). Although the vector treated mice outlived their untreated littermates, who died within 14 days of life, the benefits of the Ad vector were short-lived. The vector treated G6Pase<sup>-/-</sup> mice died after approximately 42 days, due to a rapid loss of Ad vector-mediated transgene expression. Despite the transgene expression being short-lived, the growth rates of G6Pase<sup>-/-</sup> mice increased rapidly, comparable to the growth rate of carrier and normal mice (Zingone et al., 2000). Metabolic abnormalities, such as hypoglycemia and hyperlipidemia, were corrected and excess hepatic glycogen levels were reduced. Hepatic G6Pase levels increased to 20% of the level seen in carrier mice and wild type mice (Zingone et al, 2000).

Initial studies using an AAV type 2 (AAV2-mG6Pase) vector in G6Pase<sup>-/-</sup> mice failed to fully normalize hypoglycemia or sustain life (Sun et al., 2002; Chou and Mansfield, 2007). When AAV2-mG6Pase was co-administered with Ad-mG6Pase, and two weeks later, a second infusion of the AAV2-mG6Pase vector was given, premature death was prevented and metabolic abnormalities of GSD Ia were corrected for the full 12 months of the study

(Sun et al., 2002). Although this “two vector, two step therapy” appeared to work in the G6Pase<sup>-/-</sup> mice, it was clinically irrelevant to the human model of GSD Ia due to immune reactions associated with Ad-mediated gene transfer and the resistance to the Ad vector (Chou and Mansfield, 2007). These findings suggested that while the use of a second vector administration may be efficient in providing long-term transgene expression, an improved vector-dependent treatment strategy is needed.

Another series of experiments utilized an AAV vector with different serotypes (AAV2/1-G6Pase or AAV2/8-G6Pase) to treat G6Pase<sup>-/-</sup> mice (Ghosh et al., 2006). The use of different serotypes, with distinct tissue tropisms, allowed the investigators to determine the transgene efficiency of each serotype. The AAV2/8 vector showed a clear tropism for hepatic tissue and vector-treated mice had 20.3% of normal hepatic G6Pase activity (Ghosh et al., 2006). The AAV2/1 vector delivered a functional G6Pase transgene to the liver and kidney, but vector-treated mice had only 4.3% of normal hepatic G6Pase levels. Many of the AAV1 vector-treated mice died prematurely, indicating that the hepatic G6Pase activity was inadequate for G6Pase<sup>-/-</sup> mice (Ghosh et al., 2006). The group doubly infused G6Pase<sup>-/-</sup> mice with the AAV1 vector, once at birth followed by another dose 1 week later. The method prevented premature death as vector-treated mice lived for 57 weeks with G6Pase expression in both liver and kidney. Hepatic G6Pase levels were approximately 10% of normal hepatic G6Pase levels, suggesting that levels of activity as low as this are adequate to sustain the G6Pase<sup>-/-</sup> mice for 57 weeks (Ghosh et al., 2006). Double infusion of AAV1 vector normalized serum glucose concentrations and euglycemia was maintained for the duration of the study. Hypercholesterolemia and hypertriglyceridemia were corrected by 4

weeks of age and serum cholesterol and triglyceride concentrations remained within normal limits during the 57-week study (Ghosh et al., 2006).

An AAV2 vector pseudotyped with AAV8 capsid proteins (AAV2/8) was used by Koeberl et al. (2006) to administer a canine G6Pase (c-G6Pase) transgene to G6Pase<sup>-/-</sup> mice. The pseudotyping of the AAV2 virus with the AAV8 capsid proteins allowed the vector to specifically target the liver. While this transgene normalized serum cholesterol and triglyceride levels, it only partially normalized blood glucose levels in G6Pase<sup>-/-</sup> mice in comparison to carrier and wild type mice (Koeberl et al., 2006). At 2 weeks of age untreated, affected mice weighed 50% less than clinically unaffected littermates but, after AAV2/8 gene therapy, the growth of these mice normalized to that of their unaffected littermates by 4 weeks of age (Koeberl et al., 2006). Hepatic G6Pase levels were 48% of normal G6Pase activity at 4-weeks post infusion, but declined to 21% of normal G6pase activity by 24-weeks after infusion, suggesting the AAV2/8 vector lost expression over time (Koeberl et al., 2006; Chou and Mansfield, 2007). While AAV vectors do not typically induce innate immunity, lymphocytic infiltrations were present in the liver and kidney in AAV2/8 treated mice, suggesting an inflammatory response to the AAV capsid (Koeberl et al., 2006).

Koeberl et al. (2008) pseudotyped an AAV2/8 vector encoding a human G6Pase (h-G6Pase) transgene and treated mice with this construct. Hepatic G6Pase levels remained increased for > 6 months and hepatic glycogen content was significantly reduced following vector treatment (Koeberl et al., 2008). Blood glucose levels normalized within 3 days of vector treatment and cholesterol and triglyceride levels were similar to levels observed in carrier and wild type mice.

To prevent inflammatory responses and other immune reactions associated with Ad and AAV vectors, the use of an HDAd vector, devoid of viral particles, had been instituted. Treatment of G6Pase<sup>-/-</sup> mice with an HDAd vector encoding a c-G6Pase gene reversed hypoglycemia in G6Pase<sup>-/-</sup> mice by one month of age and continued throughout the duration of the 7 month trial (Koeberl et al., 2007). Hepatic G6Pase levels were approximately 20-40% of normal at 7 months of age and hepatic glycogen content was restored to near-normal levels. Metabolic abnormalities were corrected by one month of age and were sustained through the duration of the 7 month experiment. There was no evidence of lymphocytic infiltration within the liver, indicating that the HDAd virus did not induce an immune response.

In the dog model of GSD Ia, the effects of an AAV serotype 2 (AAV2) vector was investigated in 3 GSD Ia puppies (Beaty et al., 2002). Each puppy was administered either 1.6, 7.0, or 14 x 10<sup>11</sup> vector particles on postnatal day 3. Hepatic G6Pase levels increased for all dogs, though it did not correlate to the vector dosage (Beaty et al., 2002; Chou and Mansfield, 2007). One dog, administered 7.0 x 10<sup>11</sup> particles, survived the longest (86 days) while another dog, administered 14 x 10<sup>11</sup> particles, died on day 20, and the third dog, administered 1.6 x 10<sup>11</sup> particles, died on day 39 (Beaty et al., 2002). Correction of serum cholesterol, triglyceride levels, and plasma lactate levels was observed after 8 weeks of age in the dog that lived to 86 days of age (Beaty et al., 2002; Chou and Mansfield, 2007).

The efficacy of an AAV2/8 vector carrying a human G6Pase transgene was evaluated in 3 additional dogs with GSD Ia (Koeberl et al., 2008). This vector prolonged the survival of the three dogs to > 11 months and fully prevented hypoglycemia from one month of age

(Koeberl et al., 2008). Plasma lactate and serum cholesterol and triglyceride levels for the three dogs were within the reference range for normal, unaffected dogs. Hepatic G6Pase levels were significantly elevated, equivalent to levels observed in carrier dogs. Hepatic glycogen content was significantly reduced. At one month of age, there appeared to be no difference in circulating cholesterol and triglyceride levels during fasting between dogs treated with AAV2/8, untreated, affected dogs on carbohydrate supplementation, or carrier dogs. Plasma lactate levels were reduced to levels equivalent to those observed in carrier dogs within the first month of life. Liver biopsy and histology of AAV2/8 vector-treated GSD dogs at approximately 4 months of age illustrated decreased hepatocellular lipid accumulation and vacuolation – an indication of widespread expression of the G6Pase gene throughout the liver.

While there has been extensive research of the short-term effects of vector-mediated therapies in the G6Pase mouse and dog models of GSD Ia, there is little information evaluating the long-term efficacy of these vector-mediated therapies. The key objectives of the present study were to demonstrate the efficacy of gene therapy as a long-term method of treatment of GSD Ia using the dog model and to evaluate the effects of second gene therapy treatments for correcting hypoglycemia and other biochemical and clinical abnormalities associated with GSD Ia.

## **MATERIALS AND METHODS**

### ***Breeding***

Affected puppies described in this experiment are from Malteagle (Maltese x Beagle) and/or Maltihound (Maltese x Beagle x Hound) carriers. Carrier females were checked weekly for the first signs of proestrus (vulvar swelling and discharge). When the onset of proestrus was detected, vaginal cytology was evaluated daily to determine estrus onset. Females in estrus were then artificially inseminated with freshly collected semen from carrier males via manual stimulation and were bred daily or on alternate days until the estrus period ended. Vaginal cytology was evaluated daily throughout the estrus period until signs of diestrus were prevalent. Gestation was expected to last approximately 57 days from day 1 of diestrus. Pregnancy was determined 25-30 days after day 1 of diestrus using real time ultrasonography of the abdomen. Abdominal radiographs were taken one week prior to the expected due date to determine the size and number of puppies.

### ***Neonatal Care of Puppies***

Immediately following vaginal or surgical delivery, puppies were stimulated to breathe by aspiration of mucus from the upper airway and vigorous thoracic rubbing. A small sample of blood was collected from the umbilical cord of each puppy and preserved on

a Whatman FTA card (Piscataway, NJ) for DNA analysis of G6Pase status using polymerase chain reaction.

On neonatal day 2, when puppies were stable, photographs and descriptions were made for identification purposes. Any pup showing clinical signs of hypoglycemia (i.e. CNS depression, lack of nursing) was given a 10% dextrose injection (1ml/100g) subcutaneously. Newborn puppies were monitored every 2 hours for nursing activity until genetic status was determined on postnatal day 2. Nursing, affected puppies showing clinical signs of hypoglycemia were given a subcutaneous injection of 10% dextrose (1ml/100g) and an oral milk replacement supplement every two hours (Esbilac, 1ml/100g). Body weights were collected on the day of birth and daily for the first four weeks of life. After four weeks of life, body weights were obtained monthly. Puppies were weaned at 6 weeks of age.

#### ***Polymerase chain reaction (PCR) analysis of mutation status***

PCR amplification with primers flanking the mutation site, at position 450 in the canine G6Pase cDNA, generated an 82 base pair (bp) DNA fragment. Following digestion with the restriction endonuclease, NcoI, PCR fragments were electrophoresed on a 2.0% agarose gel. NcoI digestion generated 63 bp and 19 bp fragments in a normal dog. In heterozygous carrier dogs, an 82 bp fragment representing the mutation and 63 bp and 19 bp fragments, representing the normal allele were present. In GSD affected dogs, the point mutation at position 450 eliminates an NcoI recognition site, thereby only yielding the 82 bp fragment.



### ***AAV vector preparation***

AAV vector design and production was a collaborative effort between Duke University and the UNC-Chapel Hill Gene Therapy Center as described by Koeberl et al. (2008). A PCR amplified human G6Pase promoter region (GenBank accession no. AP051355) was cloned using human genomic DNA from nucleotide position -298 to +128 using the following primers: 5'-GTGGGCCGTCTAGACTCTGTCCTGTGTCTCTG-3' and 5'-GTTGACTGGATCCCAAAGTC-3'. Using a human liver cDNA library, the human G6Pase cDNA (GenBank accession no. NM\_000151) was cloned from nucleotide position +54 to +1,186 using the following primers: 5'-CTTCCTGAGGTGCCAAGG-3' and 5'-AGATCTGCAGAATTCCTTTAAACACCGAAGACTCC-3'. The 419-base pair XbaI to BamHI genomic fragment (containing the promoter), the 1,070-base pair BamHI to EcoRI cDNA fragment, and the XbaI to EcoRI vector plasmid fragment (from pAAV-CBGAApA) were isolated (Sun et al., 2005). These restriction fragments were ligated and transformed into Sure2 competent bacteria. The resultant vector plasmid contains wild-type AAV terminal repeats flanking a G6Pase minigene, encoding human G6Pase, and a human growth hormone polyadenylation signal (pAAV-G6Pase). Bacterial colonies were column-purified and validated by sequencing the transgene, which revealed a naturally occurring, conservative substitution, Ala192Thr, in the fourth transmembrane domain, which seems to slightly enhance the effect upon G6Pase expression with AAV-G6Pase (Pan et al., 1998). The AAV2/8 vectors were pseudotyped with AAV8 capsid proteins, the AAV packaging plasmid was p5E18-VD 2/8, and the helper plasmid was pAdHelper (Koeberl et al., 2008).

All viral vector stocks were handled according to the Biohazard Safety Level 2 guidelines published by the National Institute of Health.

### ***HDAd vector preparation***

HDAd vector design and production was a collaborative effort between Duke University and Baylor College of Medicine and a gift of the Koeberl laboratory. The vector was developed using the following methods (Koeberl, personal communication). An 11-kb BamHI fragment containing the promoter/enhancer human apolipoprotein A-I (apoAI) gene subcloned into the BglIII site of modified pLPBL-1 (pLPBL-hgapoAI) was initially developed (Oka et al., 2001). A PCR amplified apoAI intron I with the following primer: 5' – CTGCGAGAAGGAGGTGCGTCC – 3' and 5' TCTCGAGTATCGATACGCGTGA ATTCGCGGCCGCGGATCCGAAGGGCCGTGGGGGAC – 3' and containing the desired enzyme sites (underlined) was created. The PCR product was digested with KpnI/XhoI, subcloned into the KpnI/XhoI site of gel-purified pLPBL-hgapoAI (phgapoAI-partial), and then subcloned into the KpnI site of phgAI-partial resulting in a plasmid containing a human apoAI promoter driven expression cassette (phgAIex). Confirmation of the 2.2-kb KpnI fragment was done by sequencing. The 0.6-kb ClaI fragment containing woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) was subcloned into the ClaI site of the phgAIex (phgAIW) while the canine G6Pase cDNA was excised by BamHI/EcoRI (Kishnani et al., 2001). The canine G6Pase cDNA was subcloned into the BamHI/EcoRI site of phgAIW (pAIW-cG6Pase) and an expression cassette containing the canine G6Pase was

excised by AscI digestion. This expression cassette was subcloned into a shuttle vector, pC4HSU (pC4HSU-AIW-cG6Pase) and was generated by subcloning a 4.4-kb BamHI fragment of cosmid C346 into pC4HSU (pC4HSU-C346) (Sandig et al., 2000). Using 293Cre66 cells, rescue and amplification of the HDAd-AIW-cG6Pase and HDAd-0 was performed and the helper virus used for amplification was AdLC9cluc (Nomura et al., 2004; Parks et al., 1996). Southern blot analysis was used to determine helper virus contamination and vector rearrangement. No helper virus contamination ( $< 0.1\%$ ) or vector rearrangement was detected in two independent vector stocks. All viral vector stocks were handled according to the Biohazard Safety Level 2 guidelines published by the National Institute of Health.

### ***Vector administration***

Following genetic analysis, GSD affected puppies were given vector therapy via jugular venipuncture at 3 days of age. Puppies were anesthetized using isoflurane given by face mask. A central venous catheterization kit with a 24-gauge catheter (AK-04650-3, Arrow International) was used and inserted into the external jugular vein. The vector was administered via the catheter, at a rate of approximately 1 mL/min while monitoring vital signs. Total injection volume depended on the puppy's weight and number of vector particles, but was usually between 3-8 mL. Following vector administration, treated dogs were monitored every 2-3 hours, 24 hours per day for clinical signs of hypoglycemia and other clinical abnormalities that might suggest hypoglycemia (lethargy, depression, vomiting,

facial twitches, etc). If clinical signs of hypoglycemia were observed, 10% dextrose (1 ml/100 g) was administered by mouth.

### ***Feeding***

All but one (Eli) of the AAV and HDAd vector-treated dogs were maintained on an 8 hour feeding regimen. Weaned dogs were offered Science Diet Puppy Dry Food (Topeka, KS) and water ad libitum throughout the day. At 7 am, 3 pm, and 11 pm dogs were offered approximately 100 grams of Science Diet Canned Food.

The feeding regimen for HDAd vector-treated Eli consisted of tube feeding (oral esophageal) 100 mL of food mixture (100 mL Clinicare and one can Iams Eukanuba Restricted Calorie Formula) every 8 hours, at 7 am, 3 pm and 11 pm.

### ***Liver Biopsy and Histology***

All vector-treated dogs had a liver biopsy taken between 4-6 months of age. One HDAd vector-treated dog, Hopscotch, had a second liver biopsy taken at 22 months of age. Dogs were anesthetized with isoflurane and a 100 mg liver sample was taken via a lateral right side laparotomy. Half of the 100 mg liver sample was flash frozen in liquid nitrogen and stored at -70° C, while the remainder was fixed in 10% buffered neutral formalin. Formalin-fixed liver samples were embedded in paraffin, sectioned at 3 to 6 microns, and stained with hematoxylin and eosin, and periodic acid-Schiff (PAS) to demonstrate glycogen.

Histology slides were examined by light microscopy by a board certified veterinary pathologist.

### ***G6Pase Enzyme and Glycogen Assay***

Hepatic G6Pase enzyme analysis was performed by using the flash frozen liver sample taken during liver biopsy as described by Brix et al. (1995). A 50 mg sample was weighed and homogenized in deionized water at a volume of 1:2 and transferred to a 15 mL conical tube. The sample was sonicated (3 x 20 secs) and centrifuged at 3000 rpm for 5 minutes. During preparation of hepatic homogenate in water, the enzyme system is disrupted and the hydrolytic activity of the membrane bound G6Pase is available for assay. The supernatant was transferred to an Eppendorf tube and centrifuged at 4°C for 3 minutes at 1000 rpm. Following centrifugation, 5 µL of homogenate was placed in 4 microcentrifuge tubes. Two of the four samples were boiled for 3 minutes to heat inactivate the enzyme. β-glycerophosphate was added (45 µL) to a non-boiled and boiled tube, while 45 µL of G6P was added to the other two tubes. Samples were incubated in a 37° water bath for 20 minutes to allow enzymatic reactions to occur. After 20 minutes, the samples were boiled for 3 minutes to stop all reactions. Samples were centrifuged at room temperature for 5 minutes at 1000 rpm. Ultraviolet cuvettes were prepared with 1.0 mL of inorganic phosphorous reagent in each. A 20 µL aliquot of the heat-inactivated homogenate samples was added to each cuvette and then incubated for 10 minutes. Samples were run on a UV spectrophotometer. G6Pase activity was measured by using G6P as a substrate after subtraction of nonspecific

phosphatase activity as estimated by  $\beta$ -glycerophosphate. G6Pase activity in each sample was normalized by protein content via the Bradford Protein Assay.

Glycogen content was measured using the homogenate produced above (Brix et al., 1995). Glycogen content was measured by complete digestion of polysaccharide using amyloglucosidase. The structure of the polysaccharide was inferred by using phosphorylase free of debranching enzyme to measure the yield of G1P.

### ***Plasma and Serum Chemistry***

Blood samples for routine serum chemistry panels and plasma lactate analysis were collected from affected dogs via the jugular vein with minimal restraint. Samples were placed in a red top tube (serum) and a purple top tube (plasma) and submitted to the clinical pathology laboratory at NC State College of Veterinary Medicine for a complete blood count (CBC) and chemistry panel. Parameters of interest were serum glucose, cholesterol and triglyceride concentrations, and lactate.

The normal canine reference range for triglyceride values and cholesterol values are 10-150 mg/dL and 125-300 mg/dL, respectively (Nelson et al., 1999). A fasted dog with a serum triglyceride concentration exceeding 150 mg/dL and a serum cholesterol concentration of 300 mg/dL is considered hyperlipidemic (Nelson et al., 1999).

Heparinized blood samples were analyzed for lactate using a blood gas analyzer (Gem Premier 3000 #14642 manufactured by Instrumentation Laboratory). The canine

plasma lactate reference range NC State College of Veterinary Medicine clinical pathology  
laboratory is 0.4 – 3.0 mmol/L.

## RESULTS

### AAV Vector Therapy in GSD Ia Dogs

An AAV2/8 vector encoding a human G6Pase transgene was administered intravenously to three GSD Ia puppies in an effort to increase survival and correct hypoglycemia and other biochemical abnormalities associated with the disease. Two of these three puppies have survived greater than 24 months (Risk and Lollipop) and the other puppy survived approximately 1 year (Jenga). Jenga met a humane endpoint during the study for recurrent hypoglycemia, despite correction of provoked hypoglycemia during routine fasting. In comparison, there was 80% mortality at two months of age of untreated, affected puppies maintained by dietary therapy (Figure 2). Risk and Lollipop continue to thrive and are currently fed every 8 hours.

AAV2/8 vector-treated dogs maintained normal fasting blood glucose concentrations, compared to untreated, affected dogs. After receiving vector on day 3 of life, blood glucose concentrations normalized for puppy, Lollipop, by 60 days of age (Figure 3). Subsequently, fasting blood glucose levels in this puppy, with one exception, have been within normal limits for >24 months. On postnatal day 43, fasting blood glucose of 185 mg/dL suggested moderately severe hyperglycemia. However, this high value more likely is the result of human or instrument error.

AAV2/8 vector injection completely corrected blood glucose values for puppy Risk (Figure 3). Before vector injection at 3 days of age, the fasting blood glucose value was 53



mg/dL. Following vector injection, fasting blood glucose levels were consistently within the normal reference range for >24 months.

The initial fasting blood glucose of puppy Jenga before vector injection at 3 days of age was 26 mg/dL. Following vector injection, fasting blood glucose values were corrected and remained normalized until this dog was euthanized on day 364 due to a failure to maintain normal blood glucose concentrations during unprovoked fasting (Figure 3).

As observed in GSD Ia human patients, AAV vector-treated GSD Ia dogs have severe growth retardation, when compared to age matched unaffected littermates (Figures 1 & 4). By two weeks of age, normal and carrier puppies were twice the body weight of vector-treated GSD Ia affected puppies. The average birth weight of the GSD Ia AAV vector-treated dogs was approximately 200 grams and by day 30, GSD Ia dogs weighed approximately 600 grams (Figure 5). Throughout the duration of the study, the three GSD Ia AAV2/8 vector treated dogs had body weights that were relatively similar (Figure 6). The average weight at the end of the study for the GSD Ia AAV2/8 vector-treated dogs was approximately 2500 grams. GSD Ia carrier dogs weights were 40-50% greater than that of the GSD Ia vector-treated dogs.

Hyperlipidemia is another hallmark of GSD Ia in humans and dogs. Previously, Koeberl et al. (2008) reported no statistically significant difference in cholesterol and triglyceride levels during fasting between the AAV2/8 GSD puppies, untreated GSD puppies on dietary therapy, or carrier dogs at 1 month of age. To evaluate AAV2/8 vector efficacy for the entire study, cholesterol and triglyceride levels for vector-treated GSD puppies were individually averaged for each GSD puppy (Figure 7). Puppy Jenga had the highest average

cholesterol (379 mg/dL), followed by Risk (372 mg/dL), and Lollipop (274 mg/dL). Jenga had the highest average triglycerides (362 mg/dL), followed by Lollipop (226 mg/dL) and Risk (124 mg/dL). A normal fasted dog with a serum triglyceride concentration exceeding 150 mg/dL (normal 10-150 mg/dL) and a serum cholesterol concentration of 300 mg/dL (normal 125-300 mg/dL) is considered hyperlipidemic (Nelson et al., 1999). This data suggested that Risk had partial correction of serum triglycerides and Lollipop had partial correction of serum cholesterol. For the duration of the study, the average (n=3) serum triglyceride concentration for all three AAV2/8 vector-treated dogs after a 2-3 hour fast was 237 mg/dL, while the average serum cholesterol concentration was 342 mg/dL.

Hyperlactacidemia is a prominent clinical manifestation of GSD Ia in humans and dogs, but not in G6Pase<sup>-/-</sup> mice. Previously, Koeberl et al. (2008) showed a decrease in plasma lactate levels in the three AAV2/8 vector-treated dogs used in this study within the first 100 days of life, comparable to levels seen in carrier dogs. However, the long term follow-up study of these vector-treated dogs revealed that plasma lactate concentrations were quite variable and elevated after 100 days of life. Plasma lactate levels for GSD puppy, Lollipop, were sharply increased initially, followed by a marked decrease. Plasma lactate levels then transiently increased until about 14 months of age (Figure 8). The canine plasma lactate reference range is 0.4 – 3.0 mmol/L, suggesting that hyperlactacidemia was present in this GSD Ia vector-treated dog for the majority of the study. GSD puppy (Risk) plasma lactate levels decreased after vector injection, followed by a transient increase until about 9 months of age (Figure 8). For the duration of the study, plasma lactate levels of GSD puppy Risk were variable but, higher than the normal plasma lactate reference range. Plasma lactate

levels for GSD puppy, Jenga, were partially corrected ( $<2$  mmol/L) during the first few months of life (Figure 8). At approximately 90 days of age, plasma lactate levels began to steadily increase and the dog became hyperlactacidemic for the remainder of the study.

Hepatic G6Pase activity was determined in tissues collected by liver biopsy performed at approximately 6 months of age from all three GSD puppies. AAV2/8 vector treatment increased hepatic G6Pase levels in all 3 GSD puppies (Figure 9). GSD puppy Risk had the lowest G6Pase level at 1.85 mmol/min/g. Jenga and Lollipop had hepatic G6Pase levels of 7.65 and 9.2 mmol/min/g, respectively. GSD Ia carrier dogs had hepatic G6Pase levels ranging from 4.9-8.0 mmol/min/g, while untreated, affected dogs had hepatic G6Pase levels ranging from 0.2-0.6 mmol/min/g. The level of hepatic G6Pase expression in the three GSD Ia vector treated dogs demonstrates a complete biochemical correction of G6Pase deficiency, which is supported by the maintenance of euglycemia.

AAV2/8 vector therapy decreased hepatic glycogen accumulation in the three GSD puppies (Figure 10). GSD puppy Risk had the highest glycogen accumulation with 1.17 mmol glucose/g protein. Interestingly, this dog had the lowest hepatic G6Pase levels. Jenga and Lollipop had lower hepatic glycogen concentrations with 0.91 and 0.51 mmol glucose/g protein, respectively. GSD Ia carrier dogs had hepatic glycogen concentrations ranging from 0.3 to 1.1 mmol glucose/g protein, while GSD Ia untreated, affected dogs had hepatic glycogen accumulation ranging from 3 to 6 mmol glucose/g protein. The low levels of hepatic glycogen accumulation seen in the AAV vector treated dogs suggest that the AAV vector was effective in delivering the functional transgene. G6Pase is a heat-labile enzyme

and low levels could also result from enzymatic degradation from inadvertent heating of the sample at any step in the G6Pase enzyme assay.

### **HDAd Vector Therapy in GSD Ia Dogs**

An HDAd vector, serotype 5 encoding a human G6Pase transgene, was initially administered intravenously to two GSD Ia affected puppies (Hopscotch, Eli). After 6 months for Eli and 22 months for Hopscotch, both of these GSD Ia dogs began showing clinical signs of hypoglycemia which was documented after short, routine fastings. Both dogs presented with anorexia and CNS depression. These findings suggested that the HDAd, serotype 5 vector lost efficacy, resulting in decreased hepatic G6Pase expression.

One dog (Hopscotch) was initially treated on postnatal day 3 with HDAd-cG6Pase serotype 5. The fasting blood glucose levels (2-3hr) for this dog indicated that after some initial fluctuation, fasting blood glucose levels normalized (Figure 11). Approximately 20 months after initial vector administration Hopscotch presented with CNS depression and anorexia. Supplemental use of dextrose on a daily basis, 3-4 times a day, and a decreased blood glucose level after 2-3 hours of fasting prompted the administration of a rescue vector at 22 months of age. The rescue vector was an HDAd-cG6Pase vector, serotype 2. The week following rescue vector administration, Hopscotch was more active, less depressed, and eating, with fasting blood glucose concentrations in a normal range, suggesting that the rescue vector had successfully transduced the hepatocytes, leading to enhanced G6Pase gene expression in the liver.

A second dog (Eli) was treated with the HDAd-cG6Pase serotype 5 vector on postnatal day 3. For the first few months of life, blood glucose levels normalized. At 6 months of age, Eli became hypoactive, depressed, and anorexic and failed to maintain a normal blood glucose level for > 4 hours (Figure 11). To maintain blood glucose levels, a 4-hr feeding schedule was implemented in an attempt to stimulate Eli's appetite. This change in feeding schedule did not reverse this dog's anorexia and proved to be ineffective at meeting the dog's nutritional requirements and maintaining euglycemia. A low-profile gastrostomy tube was inserted surgically on postnatal day 211. A rescue vector, HDAd-cG6Pase serotype 2, was administered at 7 months of age. Despite continued anorexia after vector administration, fasting blood glucose levels remained within the normal range after a 12-hr fast. Eli also became more active and appeared to have improved mental status. Increased blood glucose values and improved clinical condition suggested the rescue vector effectively delivered the transgene allowing for increased G6Pase expression in the liver.

The initial injection of the HDAd serotype 5 vector and the subsequent injection of the HDAd serotype 2 vector, prolonged survival of GSD puppy Hopscotch for >24 months and of puppy Eli for >12 (Figure 12). In comparison, untreated GSD Ia dogs typically die by 2 months of age. Both HDAd vector-treated GSD Ia dogs continue to thrive and are maintained on an 8-hr feeding regimen.

Like the AAV vector-treated dogs, HDAd vector-treated dogs had severe growth retardation when compared to their unaffected littermates. The initial body weight of the HDAd dogs was approximately 250 grams (Figure 13), approximately 50 grams more than the AAV vector-treated dogs. By 30 days of age, HDAd vector-treated GSD Ia dogs

weighed between 600 and 750 grams. The average weight at the end of the study for the HDAd vector-treated GSD puppies was approximately 3000 grams (Figure 14).

Untreated GSD puppies have marked hepatocellular swelling and vacuolation due to the intracellular accumulation of glycogen as demonstrated by PAS staining. In contrast, the two GSD puppies, Hopscotch and Eli, injected with the HDAd vector at 3 days of age had milder hepatocellular vacuolation when assessed just prior to the injection of the rescue vector. Hopscotch had relatively normal sized hepatocytes with mild vacuolation at 22 months of age while Eli had moderate hepatocellular vacuolation and glycogen accumulation at 7 months of age.

Lymphocytic infiltrates were not present in the liver of either GSD Ia puppy, suggesting that the transgene expression of G6Pase via the HDAd vector did not elicit a cellular immune response.

After initial vector injection and subsequent rescue vector injection, both GSD puppies were hyperlipidemic. The normal reference range for serum triglyceride and cholesterol is 10-150 mg/dL and 125-300 mg/dL, respectively (Figure 16). Cholesterol and triglyceride levels were individually averaged for each HDAd GSD puppy for the entire study. Hopscotch had an average serum triglyceride level of 187 mg/dL and an average serum cholesterol level of 410 mg/dL, while Eli had an average serum triglyceride level of 274 mg/dL and an average serum cholesterol level of 441 mg/dL.

HDAd vector-treated dogs also were hyperlactacidemic. Plasma lactate levels were initially increased, followed by a sharp decrease for Hopscotch (Figure 17). However, over Hopscotch's lifetime, lactate levels were highly variable. Plasma lactate levels for Eli were

initially decreased, followed by a sharp increase (Figure 17). For the duration of the study, plasma lactate levels for Eli were variable. Lactate levels seen in the HDAd vector-treated dogs were outside the reference range of 0.4 – 3.0 mmol/L, suggesting that these dogs were hyperlactacidemic despite vector administration.

Hepatic G6Pase levels for HDAd vector-treated GSD dogs were elevated when compared to untreated, affected dogs (Figure 18). At 6 months of age, hepatic G6Pase activity for the HDAd vector-treated dog (Eli) was 1.7 mmol/min/g, while it was 1.9 mmol/min/g at 22 months of age for Hopscotch. In comparison, GSD Ia carrier dogs had hepatic G6Pase levels ranging from 4.9-8.0 mmol/min/g. Untreated, GSD dogs had hepatic G6Pase levels ranging from 0.2-0.6 mmol/min/g. The increased hepatic G6Pase expression in the GSD Ia HDAd vector-treated dogs was approximately 4-fold lower than that in the AAV-vector treated dogs, suggesting that the G6Pase transgene expression was losing its efficacy in these animals.

HDAd vector injection reduced hepatic glycogen accumulation in GSD dogs (Figure 19). Hepatic glycogen concentrations in the HDAd vector-treated dogs were 0.24 and 0.37 mmol glucose/g protein. These samples were analyzed at 6 and 22 months of age. In comparison, hepatic glycogen concentrations seen in GSD Ia carriers and untreated GSD dogs were 0.3 to 1.1 and 3 to 6 mmol glucose/g protein, respectively. The low level of hepatic glycogen accumulation seen in the HDAd vector-treated dogs suggests that the initial G6Pase transgene injection was effective in delivering the G6Pase transgene.

## DISCUSSION

This study demonstrated that liver-directed gene therapy, using either an AAV or HDAd vector, successfully delivered a functional G6Pase transgene to dogs affected with GSD Ia. The effects of gene therapy using the dog model are relevant to the human model of GSD Ia because GSD Ia affected puppies have clinical abnormalities that closely resemble the neonatal onset of GSD Ia in humans (Brix et al., 1995; Kishnani et al., 2001). The 3 Maltese puppies originally determined to have GSD Ia presented with hepatomegaly, failure to thrive, poor growth, and died between 5 and 8 weeks of age (Brix et al., 1995). The clinical appearance of the affected GSD Ia dogs was consistent with the human clinical phenotype. Most noticeable in these GSD affected puppies was a distended abdomen caused by hepatomegaly as a result of the accumulation of glycogen within the liver.

The use of either the AAV2/8 or HDAd vector prolonged survival in dogs with GSD Ia. One AAV2/8 vector treated dog survived until 12 months of age and then was euthanized as a result of complications associated with the GSD Ia disease. Two AAV vector-treated dogs and one HDAd vector-treated dog have survived for >24 months and these dogs continue to thrive. The other HDAd vector-treated dog has survived to >12 months and remains in the study.

Hypoglycemia, one of the acute complications associated with GSD Ia, responded favorably to the use of either AAV or HDAd gene therapy. The prolonged prevention of hypoglycemia in AAV vector-treated GSD Ia dogs for the duration of the study, suggested that the AAV vector corrected this metabolic abnormality. Growth failure in GSD Ia humans



and animals may be linked to recurring hypoglycemia causing deficiencies in nutrients for the body. While the vector-treated dogs had decreased body weights compared to normal controls, these animals still grew. Untreated GSD dogs do not survive but if nutrient supported survival was possible, undoubtedly these dogs would weigh less than vector-treated dogs.

Other metabolic abnormalities, such as hyperlipidemia, appeared partially corrected within the first month of life in the vector-treated dogs (Koeberl et al., 2008). However, throughout the lifespan of the vector-treated GSD Ia dogs, serum cholesterol and triglyceride levels were in the upper portion of normal reference ranges, suggesting a trend towards hyperlipidemia. Despite improvement of serum triglycerides and/or serum cholesterol, vector-treated dogs continued to exhibit clinical signs of hyperlipidemia, such as abnormal fat deposition around the abdomen and upper chest.

The plasma lactate levels for the vector-treated dogs were extremely variable. The initial response to vector treatment was a decrease in lactate levels. However, following the early response to vector treatment, plasma lactate values became quite variable and were often elevated during the vector-treated dog's life, resulting in hyperlactacidemia. There does not appear to be a correlation between high lactate levels and low blood glucose levels, or vice versa. Elevated plasma lactate levels may be confounded by the stress of obtaining blood samples from puppies. During the bleeding process, there were increased respiratory rates and puppies would not remain still. This stressed state could potentially reduce the amount of oxygen in the body's tissues and cells resulting in an overstimulated glycolytic pathway. The increased glycolysis would result in increased production of pyruvate which

would be converted to lactate by anaerobic metabolism. As puppies grew older, the stress level associated with bleeding may have diminished as the puppies became more accustomed to restraint for venipuncture, resulting in plasma lactate levels that were less confounded by stress.

Hepatic G6Pase levels were corrected in the vector-treated GSD Ia dogs as evidenced by increased hepatic G6Pase activity levels in liver biopsy samples collected at 4 months of age and the prevention of recurring hypoglycemia. The increased hepatic G6Pase expression led to a decrease in hepatic glycogen storage as well in the vector-treated dogs. Two of the three AAV vector-treated dogs had approximately 50% greater hepatic G6Pase activity than the HDAd vector-treated dogs. This suggests that the AAV vector either had a higher tropism for the liver and/or greater G6Pase gene expression. This could be related to the fact that HDAd vector-treated dog G6Pase levels were taken prior to rescue vector administration for both HDAd treated dogs, suggesting that the initial G6Pase activity reflected a reduction in hepatic transgene expression. Clinical observation and fasting blood glucose levels supported this hypothesis. We anticipate increased hepatic G6Pase transgene expression and activity after rescue vector administration but our research protocol prohibited additional liver biopsies which prevented confirmation of increased transgene expression. Aside from increased G6Pase activity, all vector-treated dogs had a biochemical correction of hypoglycemia.

One of the risks associated with gene therapy is a waning effect of transgene expression over time after initial vector injection. Following initial HDAd vector injection G6Pase gene expression began to decline at 6 months for one dog (Eli) and 22 months for

another (Hopscotch). The second administration of an HDAd viral vector delivering a functional G6Pase transgene improved survival and successfully prevented hypoglycemia.

The use of AAV and HDAd vector therapies in dogs with GSD Ia greatly prolonged survival and ameliorated the metabolic abnormalities associated with the disease. The continued monitoring of these GSD Ia dogs is critical in evaluating the long-term efficacy of gene therapy treatments as there are many long-term complications associated with GSD Ia, most notably, renal failure. Regular, monthly fasts of GSD Ia vector-treated dogs should be continued to provide data for blood glucose values and serum chemistries.

Unlike the G6Pase mouse model, the characterization of GSD Ia in dogs reveals clinical abnormalities more closely resembling those associated with GSD Ia in humans. Limitations such as the absence of lactic acidosis and viability in the G6Pase mouse model make the GSD Ia dog a more favorable model. The increased size of affected animals in this model is an added benefit.

Although there are benefits of using this dog model of disease, this model provided many limitations to this study. The development of GSD Ia affected puppies relied exclusively on the successful breeding of GSD Ia carrier dogs. The number of GSD Ia affected puppies produced cannot be predicted. The dog model had many limitations within the IACUC protocol, such as the number of survival surgeries to be performed. This limited the number of liver biopsies, which provide essential information regarding vector efficacy.

The overall success of this project relies on the continued monitoring and production of GSD Ia dogs and the improvement of vector-mediated therapies. Studies evaluating different vectors, serotypes, and dosages are critical for development of a vector that

successfully corrects the GSD Ia disorder. More direct vector delivery methods (i.e. injection into the portal vein) could potentially enhance the G6Pase transgene efficiency and increase therapeutic benefits. The success of the AAV and HDAd vectors in this experiment justifies additional preclinical experiments in the anticipation that gene therapy may someday represent a curative therapeutic method in patients with GSD Ia.

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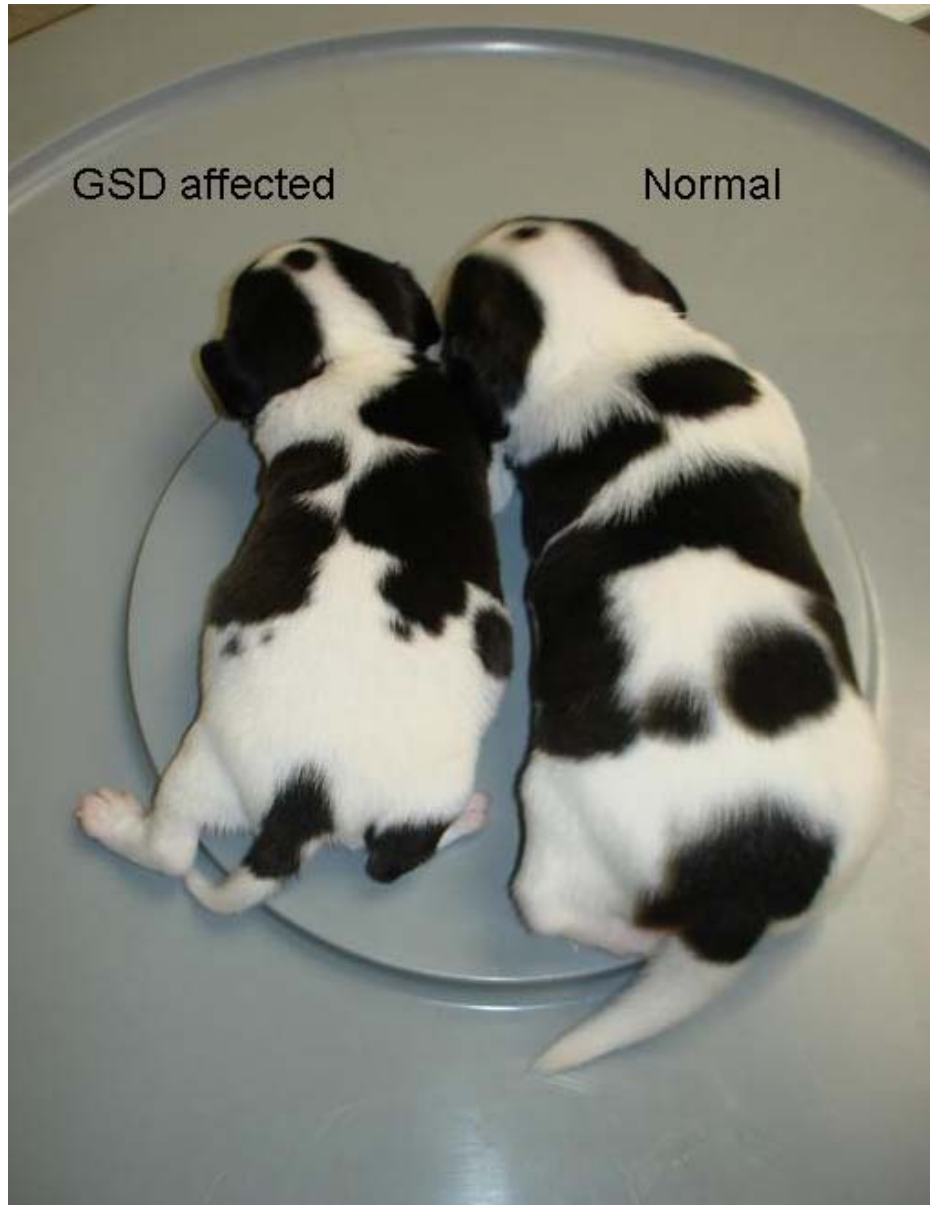
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## Common glycogen storage diseases

**Table 1:** Common glycogen storage diseases (GSDs), the deficient protein, and the primary organ affected.

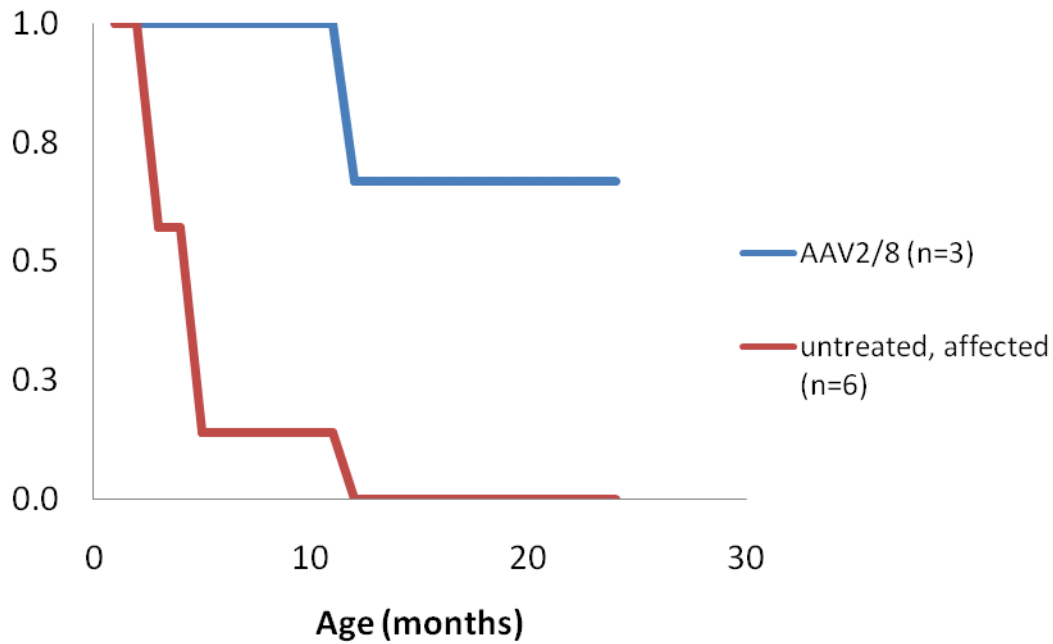
Type/Name	Deficient Protein	Primary Organ
Type Ia / von Gierke	glucose-6-phosphatase	liver
Type Ib	glucose-6-phosphate transporter	liver
Type Ic	phosphate and pyrophosphate transporter	liver
Type Id	glucose transporter	liver
Type II / Pompe	acid $\alpha$ -glucosidase	skeletal muscle
Type III / Cori or Forbes	glycogen debranching enzyme	liver and skeletal muscle
Type IV / Andersen	glycogen branching enzyme	liver and skeletal muscle
Type V / McArdle	muscle phosphorylase	skeletal muscle
Type VI / Hers	liver phosphorylase	liver
Type VII / Tauri	muscle phosphofructokinase	skeletal muscle
Type IX	phosphorylase kinase	liver and skeletal muscle
Type XI / Faconi Bickel	glucose-transporter-2 (GLUT2)	liver, kidneys, renal tubules
Type 0	glycogen synthase	liver

## GSD Ia affected dog compared to an age-matched littermate



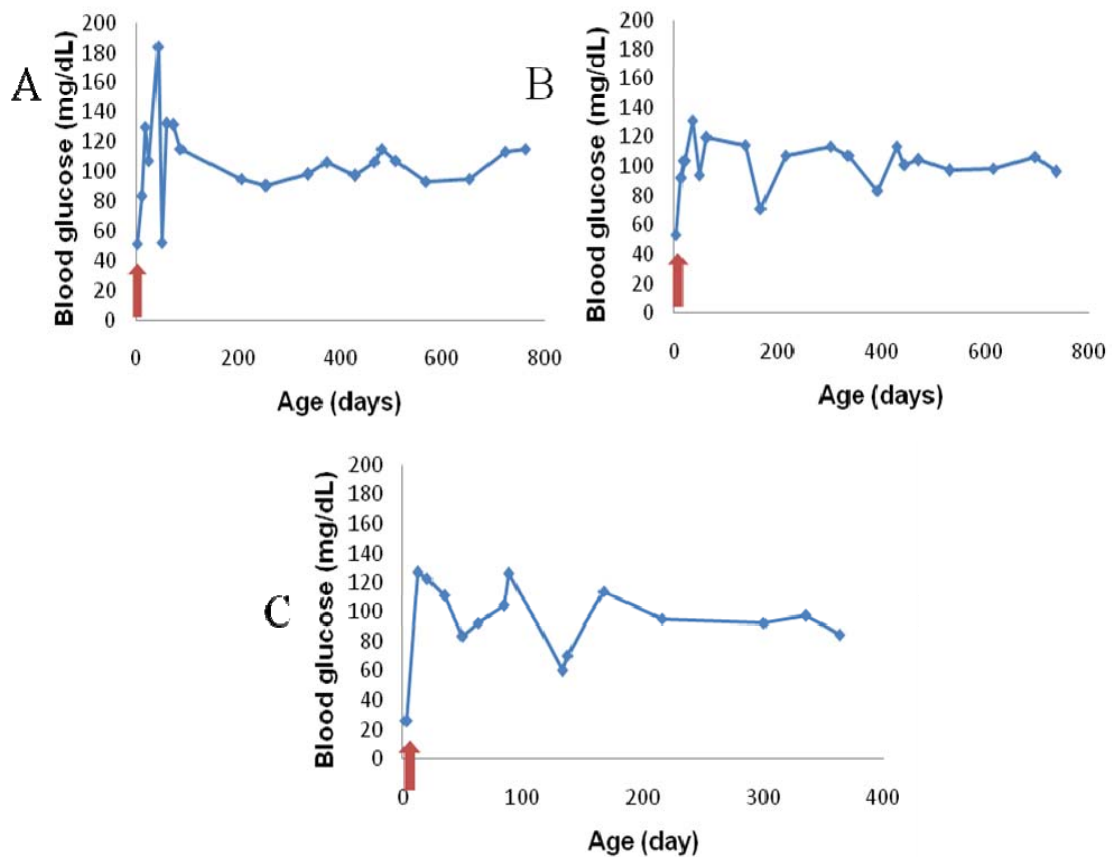
**Figure 1:** A GSD Ia affected dog is shown to the left of a normal, age-matched littermate. The affected dog demonstrates features common to this disease in dogs including decreased size and abdominal distension.

## Kaplan-Meier survival curve for AAV vector-treated dogs



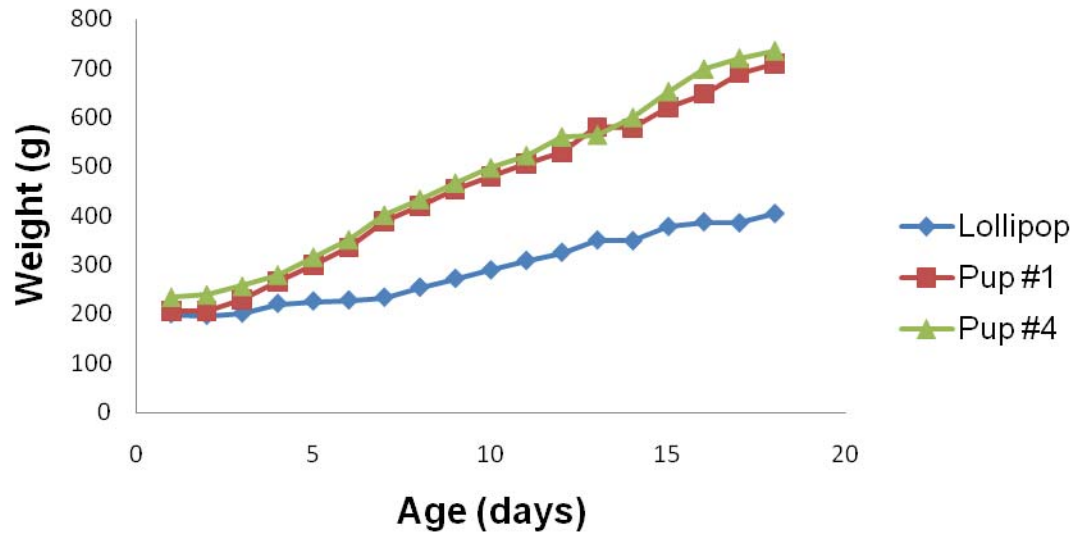
**Figure 2:** Kaplan-Meier survival of GSD Ia dogs treated with AAV/8 vector, administered at 3 days of age (n=3) and untreated control dogs (n=6). Jenga met a humane endpoint on day 364 due to an inability to maintain blood glucose concentrations during unprovoked fastings. Risk and Lollipop are >24 months of age and continue to thrive. Data for untreated, affected dogs from Koeberl et al (2008).

## Fasting blood glucose values for AAV vector-treated dogs



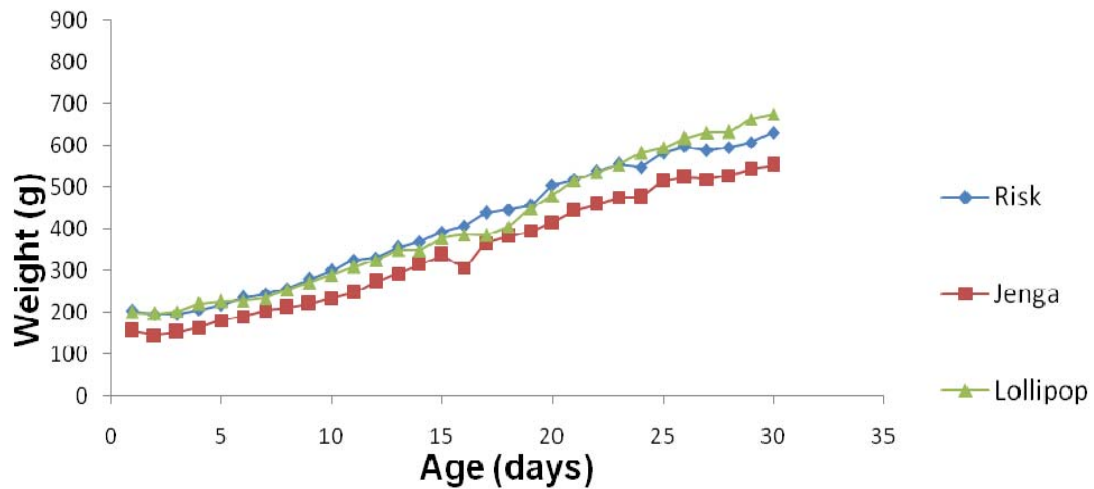
**Figure 3:** Fasting blood glucose values for AAV vector-treated GSD Ia dogs. The arrow indicates that the AAV/8-hG6Pase gene was administered on day 3 of life. After the first 2 months of life, following a 2-3 hour fast, blood glucose levels were within the normal blood glucose reference range, 53-117 mg/dL and remained normalized for the duration of the study. The dog in panel C was euthanized on postnatal day 364 due to complications associated with GSD Ia. (A = Lollipop, B = Risk, C = Jenga)

## Daily weight gain of GSD Ia affected dog compared to normal, age-matched littermates



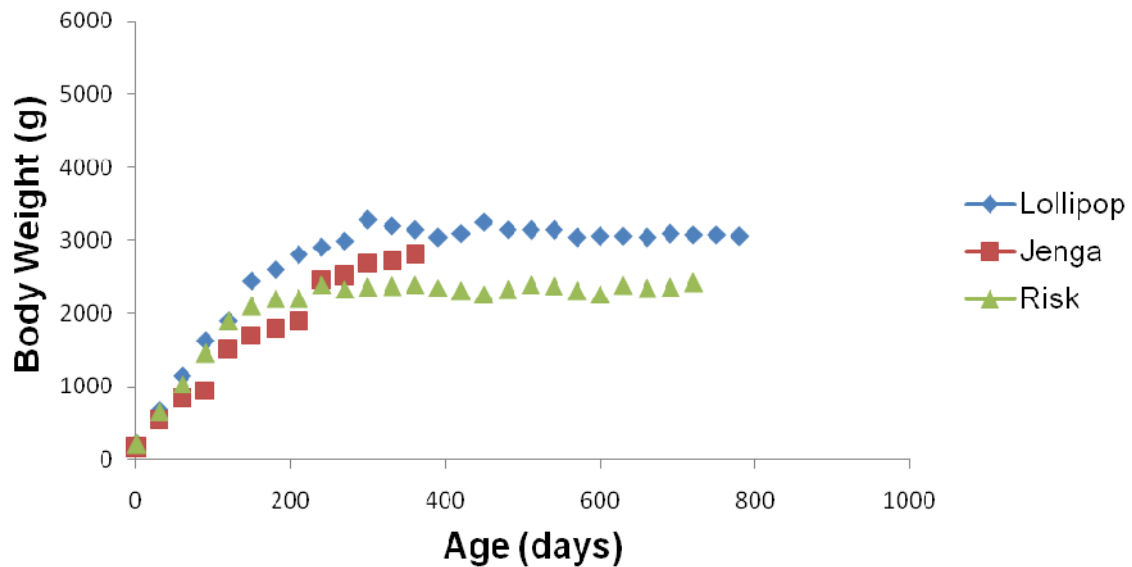
**Figure 4:** Representative daily weight gain of normal littermates vs. a GSD Ia affected dog. Unlike normal puppies, AAV vector-treated GSD affected puppies had an approximate 40 – 50% reduction in body weight gain during the first 18 days of life.

## GSD Ia AAV vector-treated postnatal body weights



**Figure 5:** GSD Ia AAV/8 vector treated postnatal body weights. The typical initial body weight of a GSD Ia affected dog is approximately 200 grams. By postnatal day 30, GSD affected dogs typically weighed close to 600 grams. In comparison, age-matched unaffected littermates weigh > 1000 grams.

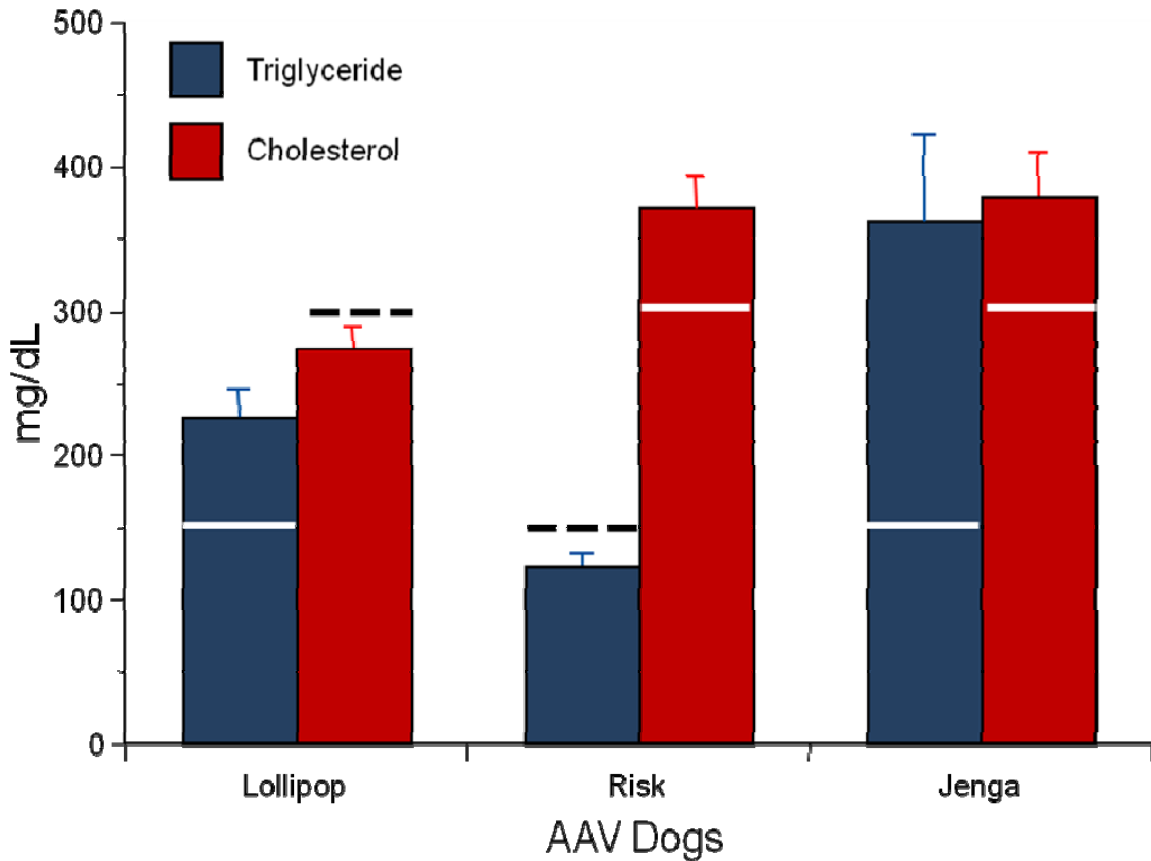
## GSD Ia AAV vector-treated body weights



**Figure 6:** Body weights of GSD Ia AAV/8 vector treated dogs through the duration of the study. Weights were taken on a monthly basis. The average adult body weight for a GSD Ia AAV/8 vector treated dog was approximately 2500 grams. In comparison, normal or carrier control dogs weigh 3000 to 6500 grams.

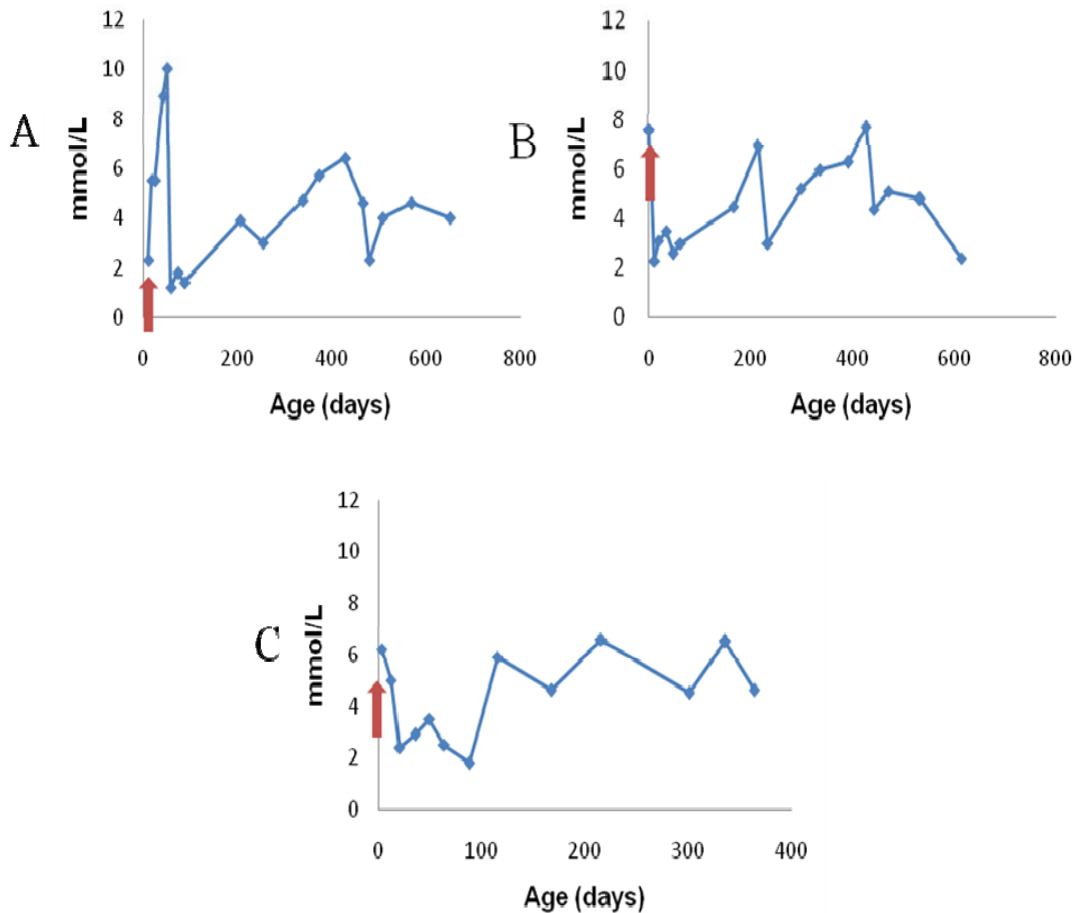


## Serum triglyceride and cholesterol levels for AAV vector-treated dogs



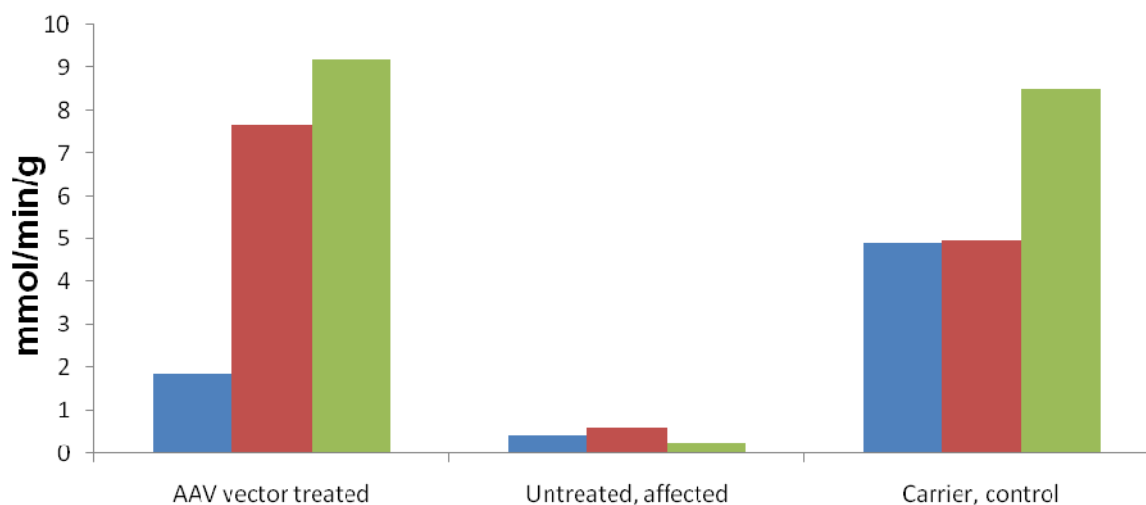
**Figure 7:** Serum triglyceride and cholesterol levels for the three AAV/8 vector-treated GSD dogs. Values shown are average triglyceride and cholesterol levels for each individual GSD Ia dog for the duration of the study. The white and dashed black lines indicate the value at which dogs are considered hyperlipidemic (cholesterol, 300 mg/dL; triglyceride, 150 mg/dL).

## Plasma lactate levels for AAV vector-treated GSD Ia dogs



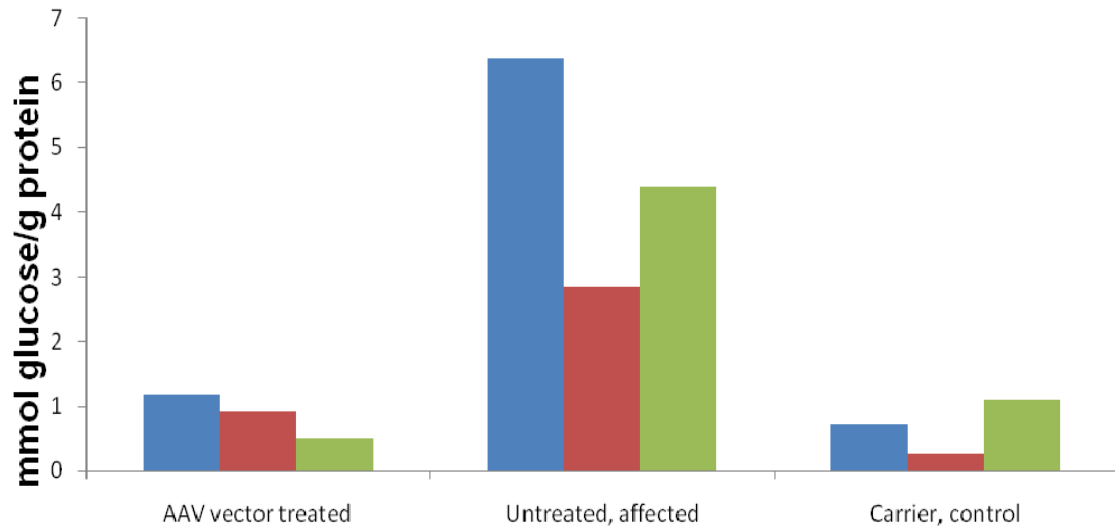
**Figure 8:** Plasma lactate levels for AAV/8 vector-treated GSD Ia dogs. The arrow indicates that the AAV/8-hG6Pase gene was administered on day 3 of life. Dog A (Lollipop) had an initial increase, followed by a sharp decrease after vector administration. Plasma lactate levels were variable for the duration of the study. Dog B (Risk) had a sharp decrease in plasma lactate levels post vector administration. Plasma lactate levels begin to rise again at approximately 180 days of age and remain variable for the duration of the study. Dog C (Jenga) had decreased plasma lactate levels until approximately 90 days of age when plasma levels began to increase. Plasma levels remained elevated for the duration of the study for all dogs, indicating hyperlactacidemia.

## Hepatic G6Pase level for AAV vector-treated GSD Ia dogs compared to untreated, affected and control dogs



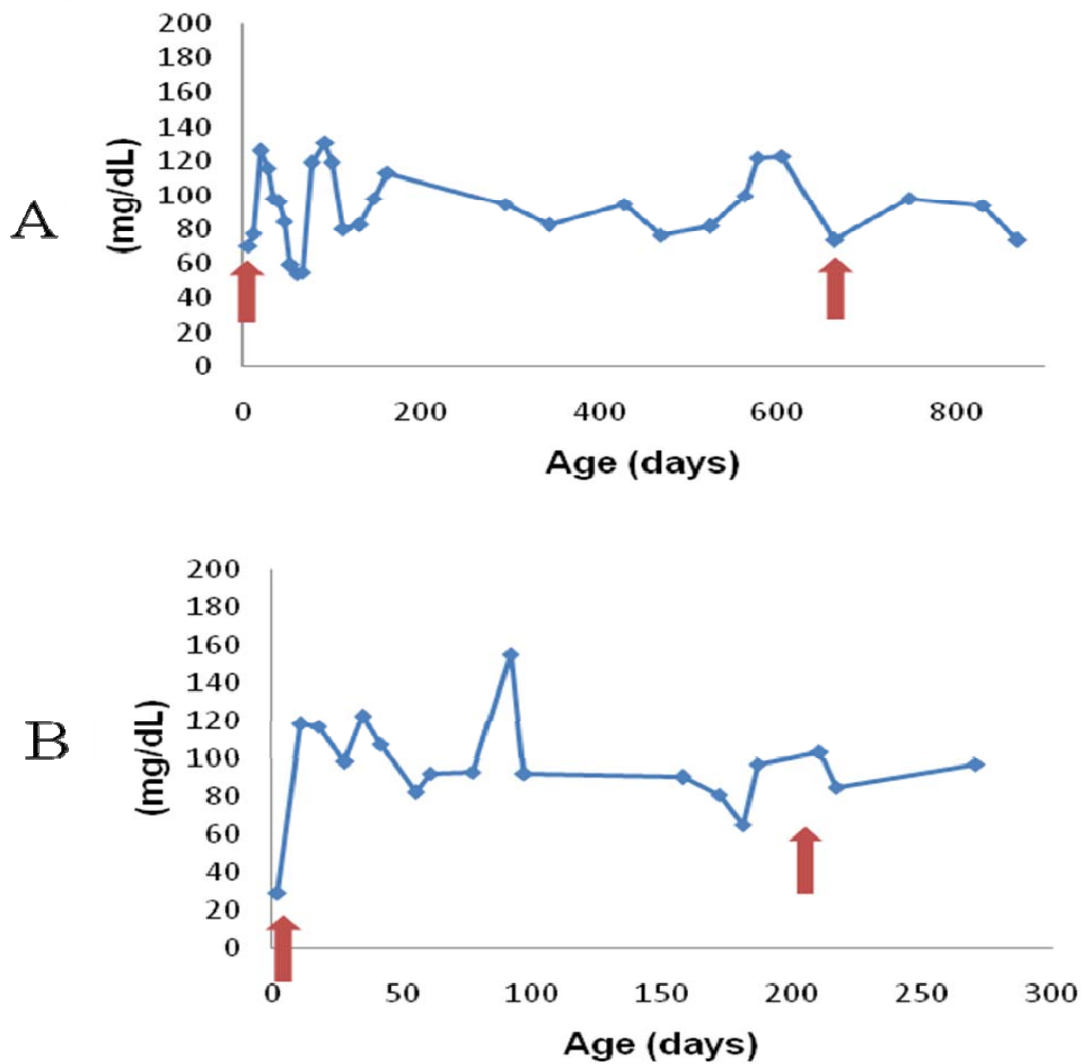
**Figure 9:** Liver biopsy-derived hepatic G6Pase levels for the AAV/8 vector-treated GSD Ia dogs (n=3), untreated affected dogs (n=3), and control dogs (n=3). Data for untreated, affected dogs and control dogs are taken from Koeberl et al. (2008). Samples from AAV vector-treated dogs were collected between 4-6 months of age.

## Hepatic glycogen content of AAV vector-treated GSD Ia dogs compared to untreated, affected and control dogs



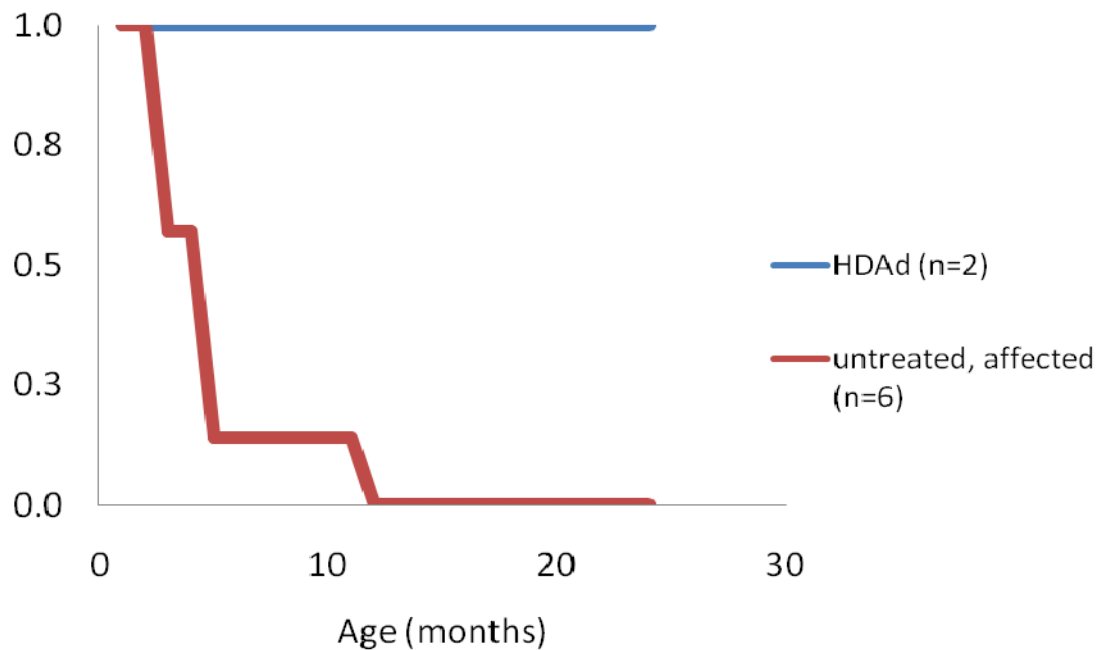
**Figure 10:** Hepatic glycogen content of AAV vector treated (n=3), untreated, affected (n=3), and control dogs (n=3). Samples from AAV vector-treated dogs were collected at 4 months of age via ultrasound-guided liver biopsy. Data for untreated, affected dogs and control dogs are taken from Koeberl et al. (2008).

## Fasting blood glucose values for HDAd vector-treated dogs



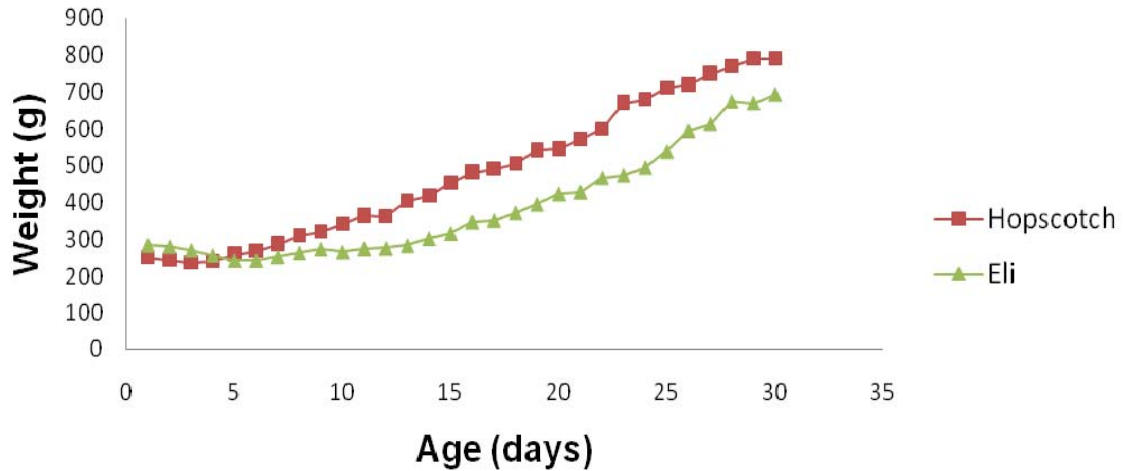
**Figure 11:** Fasting blood glucose values for HDAd vector-treated GSD Ia dogs. The first arrow indicates when the HDAd serotype 5 vector was administered on postnatal day 3. The second arrows indicates the time that the HDAd serotype 2 rescue vector was administered: panel A, postnatal day 674; panel B, postnatal day 211. (Panel A = Hopscotch, Panel B = Eli)

## Kaplan-Meier survival curve for HDAd vector-treated dogs



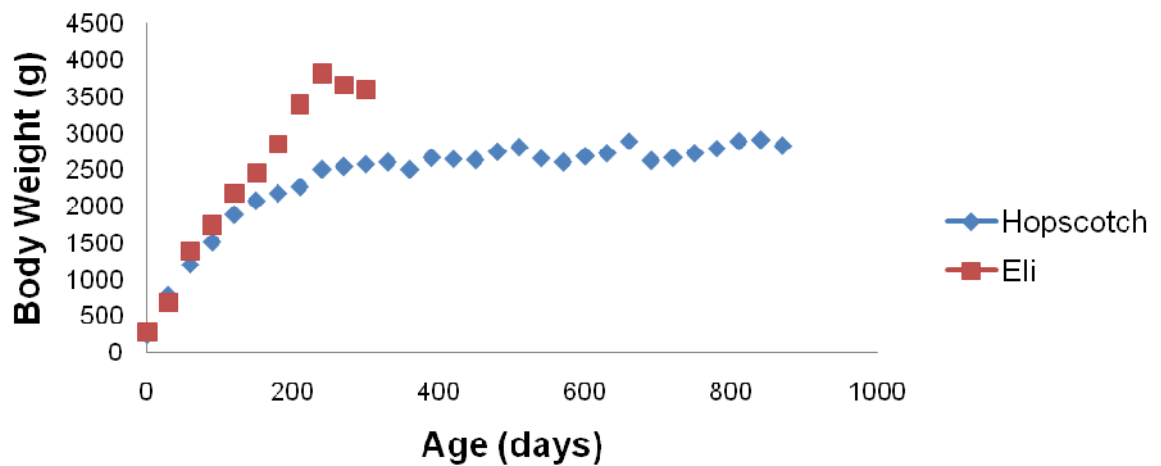
**Figure 12:** Kaplan-Meier survival of GSD Ia dogs treated with HDAd vector, administered at 3 days of age (n=2) and untreated, control dogs (n=6). At the current time Hopscotch is >24 months of age, while Eli is >12 months of age. Both GSD Ia dogs continue to thrive. Data for untreated, affected dogs from Koeberl et al (2008).

## GSD Ia HDAd vector-treated postnatal body weights



**Figure 13:** GSD Ia HDAd vector-treated postnatal body weights. The average initial body weight of a GSD Ia affected dog is 250 grams. By postnatal day 30, HDAd vector-treated GSD Ia dogs weighed between 600 and 750 grams.

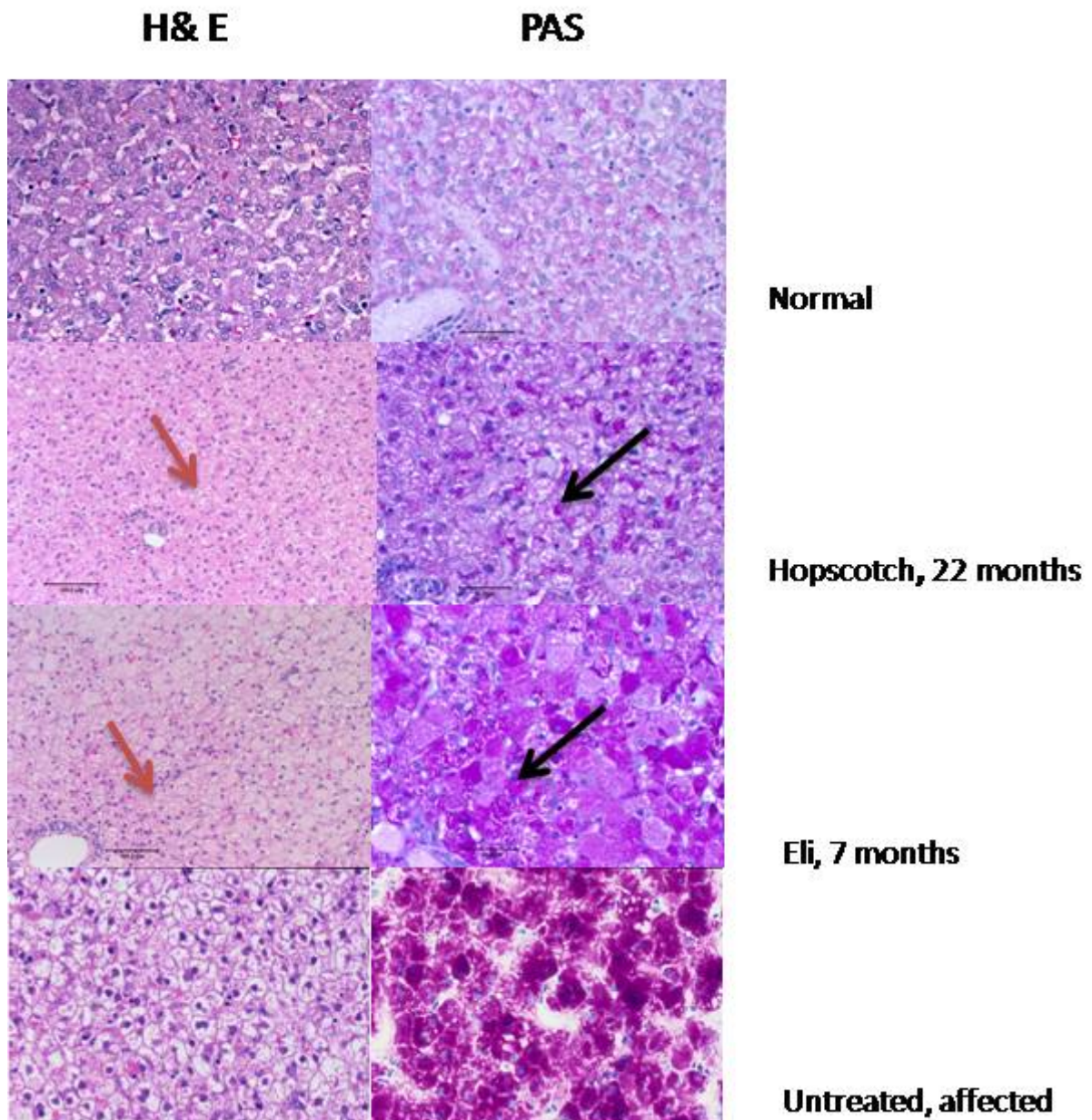
## GSD Ia HDAd vector-treated body weights



**Figure 14:** Body weights of GSD Ia HDAd vector treated dogs through the duration of the study. Weights were taken on a monthly basis. The average adult body weight for a GSD Ia HDAd vector treated dog was approximately 3000 grams. In comparison, normal or carrier control dogs weigh 3000 to 6500 grams.

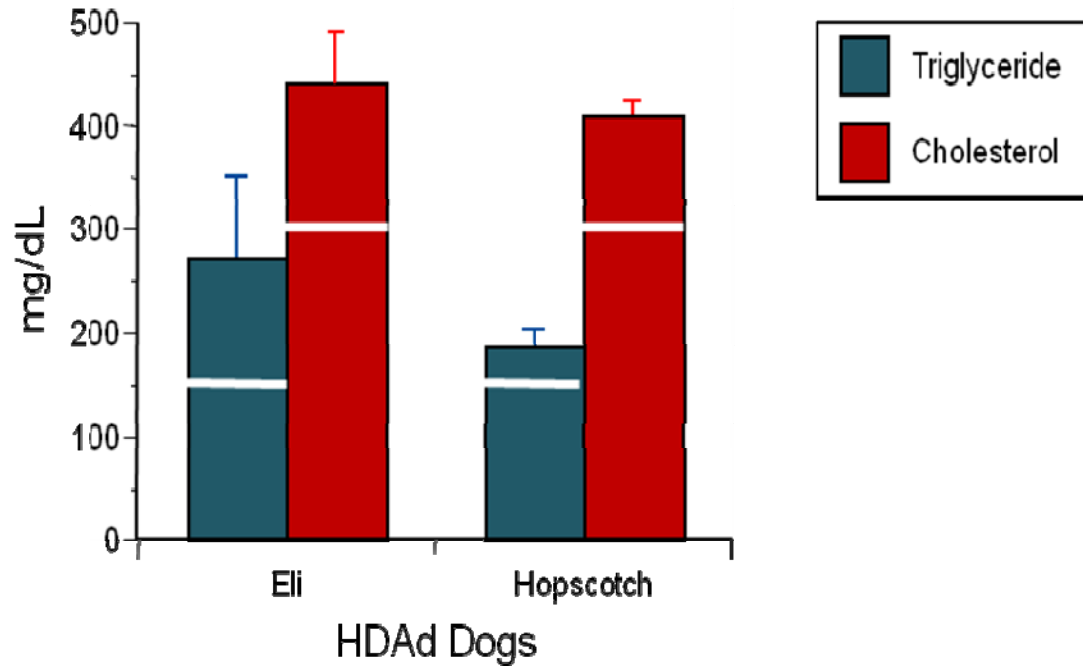


## HDAd vector-treated GSD Ia liver histology



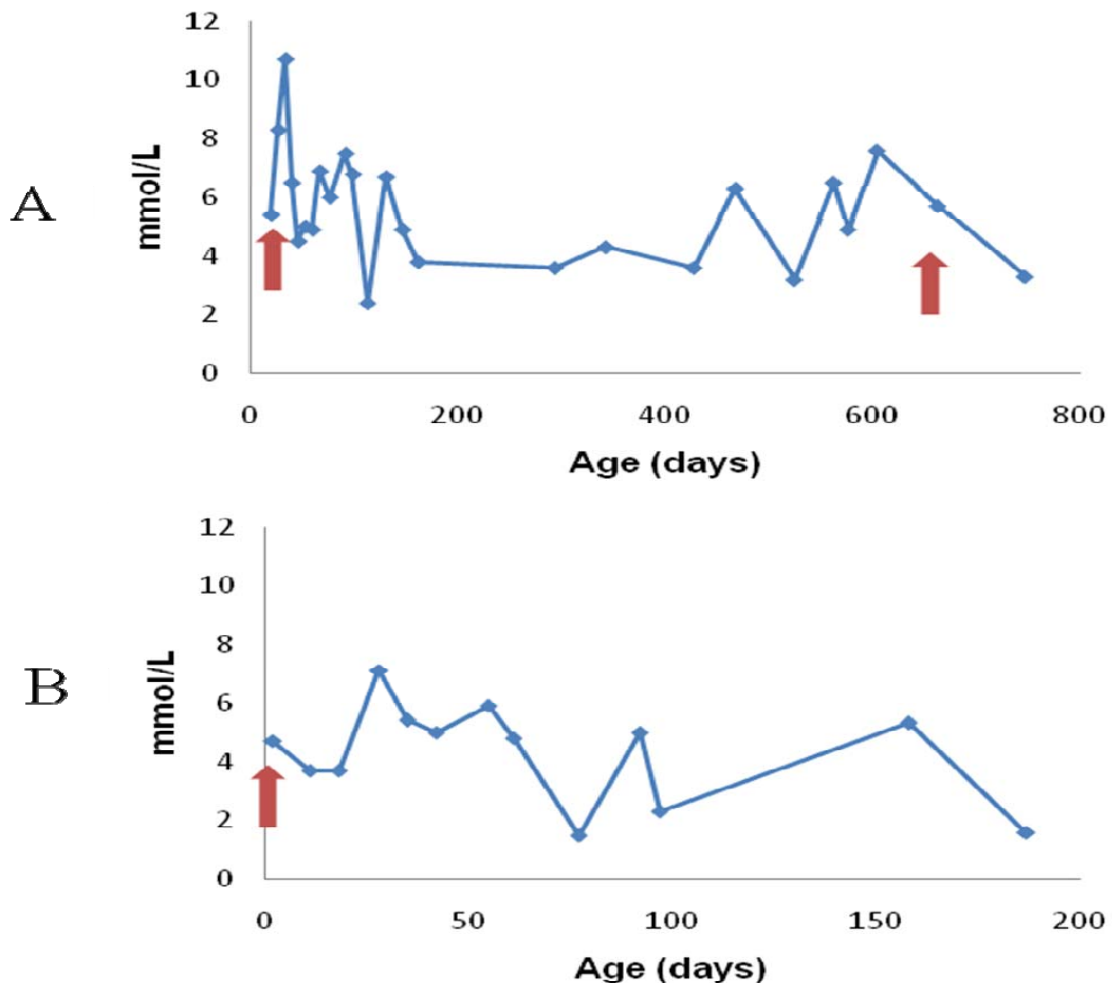
**Figure 15:** Microscopic findings seen in HDAd vector-treated GSD Ia dogs' liver. Two GSD HDAd vector-treated dogs had mild to moderate hepatocellular vacuolation (red arrow) and glycogen accumulation (black arrow) in comparison to the lack of vacuolation and glycogen accumulation in the normal dog liver and the marked hepatocellular vacuolation and glycogen accumulation in the non-treated GSD dog. H & E stains are shown at 10x magnification and PAS stains at 20x magnification.

## Serum triglyceride and cholesterol levels for HDAd vector-treated dogs



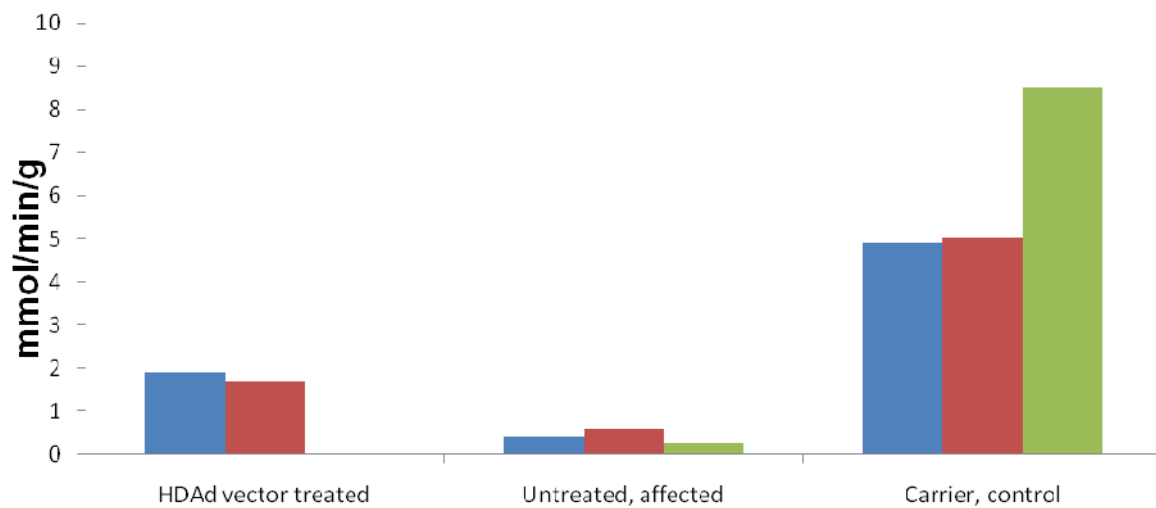
**Figure 16:** Triglyceride and cholesterol levels for the two HDAd vector-treated dogs. Values shown are average triglyceride and cholesterol levels for each individual GSD Ia dog through the duration of the study. The white line indicates the value at which dogs are considered hyperlipidemic (cholesterol, 300 mg/dL; triglyceride, 150 mg/dL).

## Plasma lactate levels for HDAd vector-treated GSD Ia dogs



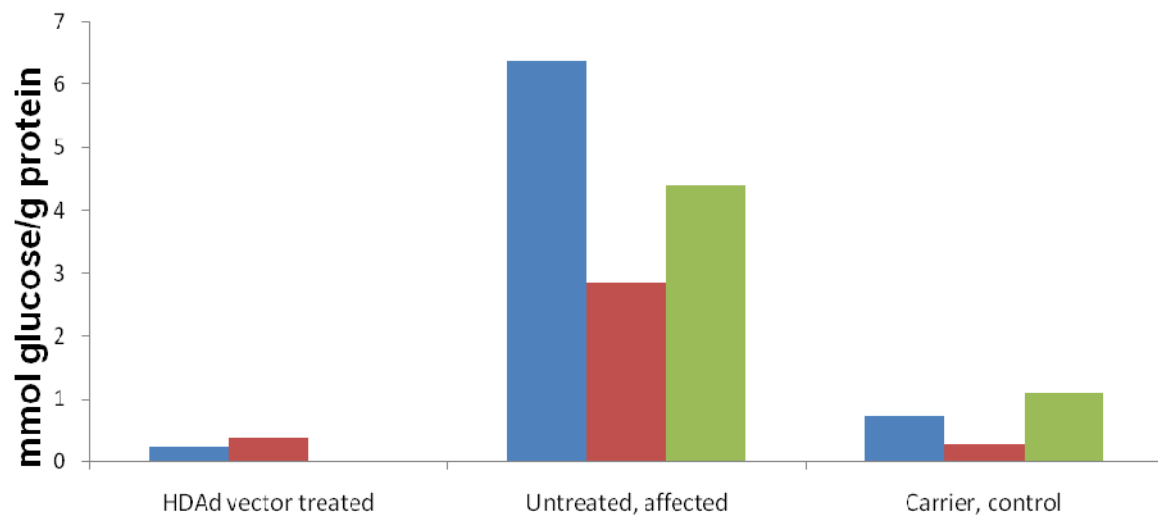
**Figure 17:** Plasma lactate levels in GSD dogs following initial HDAd vector treatment. The second arrows indicates the time that the HDAd serotype 2 rescue vector was administered: panel A, postnatal day 674; panel B, postnatal day 211. Panel A (Hopscotch) has an initial increase in plasma lactate levels, followed by a sharp decrease. Panel B (Eli) shows plasma levels have an initial decrease, followed by a sharp increase. Over the GSD Ia affected dogs' lifetime, plasma lactate values were variable and remained elevated for the duration of the study, indicating hyperlactacidemia.

## Hepatic G6Pase level for HDAd vector-treated GSD Ia dogs compared to untreated, affected and control dogs



**Figure 18:** Hepatic G6Pase activity in HDAd treated dogs (n=2), untreated affected dogs (n=3), and control dogs (n=3). The untreated, affected dog and control dog data are taken from Koeberl et al. (2008).

## Hepatic glycogen content of HDAd vector-treated GSD Ia dogs compared to untreated, affected and control dogs



**Figure 19:** Hepatic glycogen concentrations seen in HDAd vector treated (n=2), untreated, affected (n=3), and control dogs (n=3). Liver biopsy samples from the HDAd vector-treated dogs were collected at 6 and 22 months of age. The untreated, affected and control dog data are taken from Koeberl et al. (2008).