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EFFECT OF COMBINED DRUG THERAPY AND GENETIC MODIFIERS ON
HEPATOSTEATOSIS AND FIBROSIS IN A MOUSE MODEL FOR
ALSTRÖM SYNDROME

by

Ryan Hanusek

A Thesis Submitted in Partial Fulfillment
of the Requirements for a Degree with Honors
(Biology)

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Abstract

Alström Syndrome (ALMS) is a genetic disorder characterized by obesity, progressive hearing and vision loss, and insulin resistance in humans. Variability of additional phenotypes exists among patients, often complicating diagnosis of ALMS (Marshall *et al.*, 2011). Postmortem studies of patients with ALMS revealed severe fibrosis in multiple organs (Marshall *et al.*, 2011). Currently, there are no known treatments for the fibrosis observed in ALMS patients. The purpose of the studies described herein was to examine the effects of losartan, an antifibrotic drug, on fibrogenesis and to identify genomic loci that modify ALMS phenotypes in a mouse model for ALMS (*Alms1*^{Gt/Gt}).

A genome wide screen performed in two backcrosses to C57BL/6Ei and Balb/cJ mouse strains identified two genetic modifiers in *Alms1*^{Gt/Gt} mice. A significant quantitative trait locus (QTL) for high density lipoproteins (HDL) was identified on chromosome 5 in the backcross to C57BL/6Ei while significant modifying loci for retinal degeneration was identified on chromosome 2 in the backcross to Balb/cJ. Further analysis with additional progeny may reveal other significant modifiers.

Histological and chemical analyses of samples from 24-week-old mutant and control mice treated with losartan were initiated in this study. The results of this study may help future therapeutic strategies for treating human patients with ALMS.

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Introduction

Alström Syndrome (ALMS) is a rare multiple system genetic disorder (Marshall, 2005). Phenotypes associated with this disorder include: obesity, diabetes, retinal degeneration, cochlear degeneration, steatosis (the cellular retention of lipids), fibrosis, and other variable features such as hypogonadism (Collin *et al.*, 2002). Most patients do not survive past childhood as a result of cardiomyopathies or other complications. Current treatments for ALMS focus on these individual phenotypes, however some phenotypes do not currently have treatments (Marshall *et al.*, 2011). For this reason further research is required. Given the complex assembly and variability of phenotypes observed in ALMS patients that are associated with mammalian metabolism, computer models and models based on lower organisms are inadequate for understanding this disease (Hardouin *et al.*, 2000 and Bedell *et al.*, 1997). Mouse models have many characteristics making them well suited for the study of ALMS. First, mice are higher model organisms with many genes that are also found in humans. Second, their behavior and physiology have been well characterized during decades of research. Third, there are numerous issues of practicality. Mice have a relatively rapid generation time, are inexpensive to maintain, and are easy to handle. One of the world's leading suppliers of laboratory mice, and the location of prominent research in the areas of genetics and human disease, is the Jackson Laboratory located in Bar Harbor, Maine. The mouse populations maintained at the Jackson Laboratory consists of thousands of various genetic strains that are used as models for numerous human diseases. The research described herein was performed at the Jackson Laboratory.

Study Aims

One aim of this study was to identify the genetic modifiers that influence the various disease phenotypes associated with ALMS in the mouse model. To identify genetic modifiers of pathological phenotypes observed as a consequence of the disruption of the *Alms1* gene, a genome wide scan was performed in backcrossed progeny of *Alms1*^{Gt/Gt} mutants from ((C57BL/6J X Balb/cJ) F1 X C57BL/6Ei) and ((C57BL/6Ei X Balb/cJ) F1 X Balb/cJ) (Nadeau, 2003). In preliminary studies, *Alms1*^{Gt/Gt} mutants on C57BL/6Ei and Balb/cJ backgrounds exhibited different disease phenotype severities. Retinal degeneration was more rapid on Balb/cJ than C57BL/6J backgrounds (Figure 1). Additionally, ALT levels differed on both backgrounds (Figure 2). The genome scan was performed to account for any genetic differences that may cause this phenotypic variability. The computer software, Mapmanager QTX, was used to identify putative loci, which interact with *ALMS1* and may explain some of the clinical variability observed in ALMS (Manly *et al.*, 1999 and Marshall, 2005).

Since there are no known treatments for the severe fibrosis observed in multiple organs of patients with ALMS, the effects of various drugs on organ fibrosis in a mouse model for *ALMS1* were examined. The inhibition of signaling pathways in the renin-angiotensin system have been suggested as an effective therapeutic approach for other fibrotic disorders (Yoshiji *et al.*, 2001). Therefore, losartan, a drug capable of blocking the binding of angiotensin-II to angiotensin II type 1 receptor (Yoshiji *et al.*, 2001 and Bader, 2004), was administered to *ALMS1* mutant mice to determine its effectiveness in alleviating fibrosis (Figure 3). Losartan was used in combination with bezafibrate, a drug that inhibits free fatty acid composition and promotes beta-oxidation, to diminish high fat

levels observed in mutant mice (Figure 4) (Goldenberg *et al.*, 2008). The identification of genetic modifiers and drug treatments for Alström Syndrome may eventually provide more treatment options to patients, and provide more information for related diseases such as diabetes, obesity, and liver disease.

Diagnosis

Diagnosis and treatment of ALMS is difficult because of its shared similarities to other disorders, such as the Bardet-Biedl Syndrome (Marshall *et al.*, 2007), and of the differences in onset, progression and absence or presence of the associated phenotypes (Marshall *et al.*, 2005). These phenotypic differences among patients may be due to genetic background modifiers as well as the influence of environmental factors. Blood, vision, and auditory tests as well as heart and thyroid assessments may provide information regarding the diagnosis of ALMS. The diagnostic criterion for ALMS consists of the presence of some of the cardinal features of the disease and genetic testing. The cardinal features of the syndrome include neurosensory loss, obesity, cardiomyopathy, organ fibrosis, steatosis, insulin resistance and its associated complications. Neurosensory loss often manifests at birth with sensitivity to light and reduced visual acuity (Marshall *et al.*, 2005). Visual degeneration progresses into adolescence at which point most patients are legally blind (Marshall *et al.*, 2005). Studies have found degeneration of the retinal pigment epithelium, cone-rod dystrophy, weakened vessels, and subcapsular cataracts in patients (Marshall *et al.*, 2005). Auditory impairment often manifests in childhood and may progress to deafness. Many patients also exhibit dilated cardiomyopathy in infancy through childhood, or restrictive cardiomyopathy in adolescence through adulthood, which in some cases proves fatal.

Hepatic dysfunction, which is often observed, can range from elevated transaminases to overt cirrhosis (Marshall *et al.*, 2007). Liver biopsies showed signs of inflammation and steatosis, or abnormal retention of cellular lipids (Marshall *et al.*, 2005). Pulmonary complications include asthma, bronchitis, inflammation, and hypoventilation.

Postmortem studies in patients with ALMS showed severe fibrosis in multiple organs including the heart, lungs, and liver (Marshall *et al.*, 2005). These phenotypes and the approximate age of appearance within the patient are represented in Figure 5.

Treatment

Treatment for ALMS is restricted to the individual treatments of its phenotypes. The rates of progression, age of onset, and degree of severity for retinal degeneration, cochlear degeneration, hepatic dysfunction, renal dysfunction, and cardiomyopathy have not yet been fully elucidated nor have treatments altered their progression (Paisey, 2009). However, patient outcomes can be improved with early detection, monitoring, and intensive medical intervention (Marshall *et al.*, 2005). In all cases, retinal degeneration progress to total blindness by adolescence or adulthood (Marshall *et al.*, 2007). Patients are given mobility training at an early age, and hearing aids are utilized to help manage hearing loss (Marshall *et al.*, 2007). ALMS patients with type 2 diabetes are restricted to low carbohydrate diets to decrease serum glucose and insulin levels (Paisey, 2009). Patients with high lipid levels are treated with insulin therapies, dietary restrictions, and nicotinic acid to decrease triglyceride synthesis (Jabbar *et al.*, 1998 and Paisey, 2009). There are currently no known treatments for systemic fibrosis observed in ALMS patients.

Alström Syndrome Genetics

ALMS is a progressive autosomal recessive genetic disease; therefore, the parents of afflicted patients must each carry a recessive copy of the allele and pass it on to their offspring in order for the disease phenotype to be observed. The heterozygous carriers of the recessive allele do not show any outward signs of the mutation, but genetic testing can reveal carriers. In humans, nonsense or frameshift mutations in exons 8, 10, and 16 of the *ALMS1* gene on chromosome 2, position 13 are attributed as the cause of ALMS (Hearn *et al.*, 2002). Although mutations in any of these exons have been linked to ALMS, mutations in exons 8 and 16 have been shown to be more frequent (Figure 6). Translation of the normal *ALMS1* gene results in a 4169 amino acid protein that is ubiquitously expressed and found as part of the centrosome (Andersen *et al.*, 2003 and Marshall *et al.*, 2011).

ALMS Mouse Model

The ALMS mouse model, *Alms1*^{Gt(XH152)Byg} herein referred to as *Alms1*^{Gt/Gt}, is a genetic mouse strain created and maintained at the Jackson Laboratory prior to the start of this experiment. This mouse model was generated by microinjecting embryonic stem cells containing a genetrapp cassette into the blastocysts of C57BL/6J wild type mice (Collin *et al.*, 2005). The resulting chimeric males were then backcrossed with C57BL/6J wild type mice and the resulting progeny were then genotyped to identify wild type (*Alms1*^{+/+}) and heterozygous (*Alms1*^{Gt/+}) mice. Identified heterozygous mice were then bred to generate *Alms1*^{Gt/Gt} mutant mice. This method of generating *Alms1*^{Gt/Gt} mice was devised as a result of difficulties breeding *Alms1*^{Gt/Gt} mice due to obesity complications. The *Alms1*^{Gt/Gt} mouse model exhibits similar characteristics of the human disease such as

obesity, hepatic disease, hypogonadism, hyperinsulinemia, retinal dysfunction and hearing loss (Marshall *et al.*, 2011) (Figure 7). Due to the gene-trap cassette insertion in intron 13 of the *Alms1* gene, this mouse mutation results in an aberrant splicing into the cassette resulting in a prematurely terminated protein at the 3' end of the gene (Marshall *et al.*, 2011). This mutation is similar to the most common mutations in human ALMS patients, which also results in a prematurely terminated protein, with variable retention of function, at the 3' end of the gene.

Significance:

The hepatic dysfunction observed in ALMS patients shares many characteristics that are also common to non-alcoholic fatty liver disease and non-alcoholic steatohepatitis (NAFLD/NASH), namely, steatosis, hyperinsulinemia, hypertriglyceridemia, inflammation, and fibrogenesis (Matteoni *et al.*, 1999). Both of these diseases, and their individual phenotypes, are more commonly found in developed countries where high calorie, sugar, and fat diets are more common (Sanyal, 2002). It is estimated the prevalence of NAFLD in these countries is approximately 20% and that of NASH to be approximately 3-5% with both on the rise (Targher *et al.*, 2007). The development of non-alcoholic steatohepatitis (NASH) occurs in approximately 25% of patients with non-alcoholic fatty liver disease (NAFLD), a common form of chronic liver disease (Targher *et al.*, 2007). Necroinflammatory changes and pericellular fibrosis distinguish NASH, the more severe form of the disease from NAFLD, which is associated with visceral obesity and insulin resistance (Matteoni *et al.*, 1999). Patients with NASH are at higher risk for developing advanced fibrosis, cirrhosis and hepatocellular carcinoma, for which current treatments—diet, increased physical activity,

antioxidants, lipid-lowering agents, and insulin sensitizers—have had little histological impact on the liver (Sanyal, 2002). Currently, the mechanisms that influence the transition from NAFLD to NASH are not fully understood. It is hypothesized that hypertriglyceridemia and an associated accumulation of liver triglycerides from a high fat diet in conjunction with inflammatory responses to environmental factors and/or a genetic predisposition causes the observed fibrogenesis (Wood *et al.*, 2008). Recent studies have identified an increase in transforming growth factor (TGF- β) and procollagen I mRNA levels in diet induced NASH mouse models (Hirose *et al.*, 2007). Angiotensin receptor blockers have been shown to delay the progression of hepatic fibrosis and also decrease TGF- β and collagen gene expression. Losartan, a selective angiotensin II type I receptor antagonist, is capable of reducing hepatic fibrosis in certain human and animal models (Yokohama *et al.*, 1980). Studies involving the drug bezafibrate have resulted in reduced accumulation of fat and improved diet induced insulin resistance (Sasaki *et al.*, 2011). Due to the similarities between the hepatic pathogenesis in NASH/NAFLD and ALMS, the dissection of genetic modifiers of hepatosteatosis and fibrosis in the *Alms1*^{Gt/Gt} mouse model may provide further insight into the biochemical pathways involved in both conditions. Further research into the effects of bezafibrate and losartan on the hepatic disease in the *Alms1*^{Gt/Gt} model will provide valuable information leading to treatment options for ALMS, NAFLD/NASH, and the phenotypes found in both diseases for human patients.

Methods

The experimental methods described herein, excluding routine mouse care, were performed by me, under the supervision of Gayle Collin and Dr. Jürgen Naggert. Experimental aims and designs were planned prior to my involvement with the project. Histological sectioning and DNA sequencing was performed by their respective laboratories at The Jackson Laboratory.

Mouse Care:

All mice were housed in Dr. Jürgen Naggert's animal room at Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained in sterile barrier facilities according to Jackson Laboratory institutional animal care and use (ACUC) procedures. Personnel were required to wear sterilized clothing, gloves, shoes, caps, and masks. Mice were kept in microisolator caging with sterilized bedding, irradiated feed, and filtered and acidified drinking water. Mice were transferred to clean cages using disinfected forceps.

Losartan and Bezafibrate Experimental Design:

Alms1^{Gt/Gt} mutants and wild type controls were identified by amplification using the polymerase chain reaction (PCR) (Collin *et al*, 2005) on DNA isolated from Proteinase K-digested tail snips. Losartan and bezafibrate were given at sixteen weeks of age for eight weeks. Four experimental trials were constructed. Trial one served as a control group in which *Alms1*^{Gt/Gt} mutant and control mice did not receive drug treatment. Trial two consisted of *Alms1*^{Gt/Gt} mutant and control mice treated with losartan. In trial three, *Alms1*^{Gt/Gt} mutants and controls were treated with both losartan and bezafibrate. The fourth trial consisted of *Alms1*^{Gt/Gt} mutants and controls treated with bezafibrate. Mice did

not exceed five individuals per cage. Blood plasma draws were taken for all groups prior to treatment at sixteen weeks of age, at twenty weeks of age and at twenty-four weeks of age. At twenty-four weeks of age, or after eight weeks of treatment, mice were sacrificed, organs removed, and organ histopathology analyzed.

Drug Administration:

Losartan, supplied by Merck & Co. (Whitehouse Station, NJ), was dissolved in 1ml UltraPure Distilled Water at a concentration of 0.3 g/ml. Losartan was further diluted with distilled water and administered to mice of all trials at a concentration of 300 mg/L. Dosage levels of losartan were based on earlier titration studies in previously published mouse studies (Habashi *et al.*, 2006). Each mouse was estimated to drink 1.3ml /10g body weight. The actual amount of losartan consumed was determined by monitoring the volume of water consumed weekly. Losartan-treated water was changed weekly to prevent microbial growth.

Bezafibrate was administered in irradiated standard food diet at 1g/kg prepared by Research Diets (New Brunswick, NJ) such that each animal received 100mg/kg/day. The daily dose of bezafibrate per cage was determined by the equation: $DD = SD$ (100mg/kg/day) X average body weight (g)/average daily food intake.

Chemical Analysis:

Monthly blood plasma draws and body weights were taken to monitor metabolic parameters throughout the course of drug treatment. Blood samples were taken just prior to treatment at sixteen weeks of age, at twenty weeks of age, and twenty-four weeks of age. Trans-orbital eye bleeds were performed on mice according to ACUC-approved

protocol at the Jackson Laboratory (LAH 92-05). Blood plasma was isolated by centrifugation at 12,000 rpm at 4°C for 5 minutes and sent for chemical analysis at the Jackson Laboratory. HDL, total cholesterol, triglycerides, free fatty acid, ALT, AST, and blood glucose levels were assessed using the Beckman Synchron DXC 800 (Beckman Coulter, Inc.).

Insulin (Mouse) High Range ELISAs (ALPCO Immunoassays, Salem, NH) were performed according to manufacture's protocol. Blood plasma was isolated by centrifugation at 12,000 rpm at 4°C for 5 minutes. Insulin levels were analyzed sixteen, twenty, and twenty-four weeks of age for mutant and control mice using a Mouse Insulin ELISA kit (ALPCO). All samples were analyzed in duplicates and the absorbencies averaged. Sample absorbance was measured at a wavelength of 450nm using a SpectraMax 190 (Molecular Devices, Inc., Cilicon Valley, CA) in conjunction with ALPCO supplied insulin standards.

Histological Analysis:

The extent of fibrosis and steatosis was assessed by histological evaluation of Sirius Red and Hematoxylin and Eosin (H&E) stained, Bouin's-fixed liver and kidneys, respectively. After eight weeks of treatment, mice were sacrificed by CO₂ euthanasia and the eyes, liver and kidneys removed. The eyes were then fixed overnight in cold methanol, acetic acid, and PBS in a 3:1:4 dilution and the liver and kidneys in Bouin's fixative. The kidneys were then weighed to determine changes in response to fibrosis and steatosis. Fixed tissues were embedded, sectioned, deparaffinated and stained. Kidneys and eyes were stained with hematoxylin and eosin, and livers with Sirius Red. Images were then taken using a digital microscope camera with a Leica DM LB

microscope (Meyer Instruments, Inc., Houston, TX) and comparisons were made between control and mutant mice. Kidneys were imaged to monitor if any further changes resulted from the losartan treatment.

Genomic Modifier Mapping Experimental Design:

To identify quantitative trait loci (QTL) that may interact with *Alms1* to modify the ALMS phenotypes, we selected two background strains that carried the *Alms1* mutation: C57BL/6Ei- *Alms1*^{Gt/Gt} and Balb/cJ- *Alms1*^{Gt/Gt}. Two different sets of backcrosses were bred: (B6 X Balb- *Alms1*^{Gt/+})F1 X Balb-*Alms1*^{Gt/+} and (B6-*Alms1*^{Gt/+} X Balb)F1 X B6-*Alms1*^{Gt/+}. *Alms1*^{Gt/Gt} genotypes were identified upon weaning, as previously described, and DNA from homozygous *Alms1*^{Gt/Gt} mutants were selected for the genome scan. *Alms1*^{Gt/Gt} DNA was amplified by PCR using evenly spaced microsatellite repeat markers across the genome. PCR results were separated based on different backcrosses: (B6 X Balb -*Alms1*^{Gt/+})F1 X Balb-*Alms1*^{Gt/+} and (B6-*Alms1*^{Gt/+} X Balb)F1 X B6-*Alms1*^{Gt/+}. Mapmanager QTX computer software was then used to identify linkages between genotype and phenotype.

Determination of Alms1 Genotype:

DNA was isolated from tail snips of (B6-*Alms1*^{Gt/+} X B6-*Alms1*^{Gt/+})F2 progeny using proteinase K digestion and salt precipitation (Strauss, 1998). Genotypes of *Alms1*^{Gt/Gt}, heterozygous, and homozygous wild type mice were differentiated through amplification of isolated DNA with allele-specific primers. Cycling conditions for genotyping of isolated DNAs were as follows: 94°C for 2 min followed by 45 cycles of 94°C for 20 sec, 54°C for 30 sec, and 72°C for 40 sec. A final extension was done at 72°C for 7 min. PCR reactions were done with two different primer sets for each DNA sample to test for

the wild type and mutant alleles. Reactions took place in a 11 μ l volume containing 10X PCR reaction buffer (Boehringer Mannheim), isolated genomic DNA, 25 mM MgCl₂, 10 mM dNTPs, and 0.25U Taq polymerase (Roche). For testing the wild type allele 10 μ M primer 13.3F (5'-tgcaattatgcctgcatgc-3'), and 10 μ M primer 13.2R(5'-agccttccccaggtcaac-3') were added. For testing the gene trapped allele 10 μ M primer 13.2F(5'-gatcttatgagtcacccatag-3') and 10 μ M primer En2.4R(5'-aacgggttcttctgtagtcc-3') were added. DNAs were separated on a 1.5% agarose gel, stained with ethidium bromide, and examined under UV light. The presence or absence of bands from both primer sets determined the genotype.

Genomic Modifier Mapping:

DNA was isolated from tail snips of (*B6 X Balb -Alms1^{Gt/+}*)F1 X *Balb-Alms1^{Gt/+}* and (*B6-Alms1^{Gt/+}* X *Balb*)F1 X *B6-Alms1^{Gt/+}* backcrossed progeny as described above. *Alms1^{Gt/Gt}* DNAs were amplified with 65 microsatellite repeat markers spaced, on average, 20 cM across the genome. Locations suggestive of linkage with the quantitative phenotype were further tested with markers < 20 cM spacing. Cycling conditions for amplification of DNA specified by the microsatellite repeat markers were as follows: 94°C for 2 min followed by 49 cycles of 94°C for 20 sec, 50°C for 30 sec, and 72°C for 30 sec. A final extension was done at 72°C for 7 min. Reactions took place in a 11 μ l volume containing 10X PCR reaction buffer (Boehringer Mannheim), isolated genomic DNA, 25 mM MgCl₂, 10 mM dNTPs, 0.25U Taq polymerase (Roche), and 10 μ M of forward and reverse primer. DNAs were separated on a 4% metaphor/agarose gel, stained with ethidium bromide, and examined under UV light. Results were interpreted

when compared with C57BL/6Ei and Balb/cJ controls. DNAs were also sequenced and entered into Mapmanager QTX for analysis.

Statistical Analysis:

Identification of putative modifying loci was detected using the Mapmanager QTX computer software. Map Manager identifies QTLs through five levels of complexity. Associations were made between trait values and genotypes, hypothetical QTLs, background modifiers, and composite interval mapping (Manly *et al.*, 1999). Permutation tests were then performed using Map Manager.

Results

Interpretation of experimental data was done under the guidance of Gayle Collin.

Drug Administration:

Alms1^{Gt/Gt} and control littermates' body weights and losartan consumption were measured over the 8-week experimental period (Figure 9). Normalized, average body weight and data were observed to be proportionally higher for group 1 than group 2; group 2 consisted of three mice, one *Alms1^{Gt/Gt}* and two *Alms1^{Gt/+}*, and group 1 consisting of four mice, three *Alms1^{Gt/Gt}* and one C57BL/6Ei wild type. Analysis of the data indicated the one *Alms1^{Gt/Gt}* mouse within group 2 was the heaviest mouse in the study by approximately 3 grams, and the heaviest mouse within group 2 by approximately 18 grams. However, it was the only *Alms1^{Gt/Gt}* mutant in group 2.

Chemical analysis:

To determine whether losartan could influence phenotypes observed in *Alms1^{Gt/Gt}* mice, blood samples were taken at 16 weeks and 24 weeks of age, before, after, and without treatment with losartan. Blood serum parameters observed in *Alms1^{Gt/Gt}* and wild type groups both before and after losartan treatment are shown in Table 1.

Table 1. Clinical Chemistry Analysis of Losartan Administered *Alms1^{Gt/Gt}*:

<i>Parameters</i>	<i>controls</i>	<i>Alms1^{Gt/Gt}</i>	<i>controls</i>	<i>Alms1^{Gt/Gt}</i>	<i>controls</i>	<i>Alms1^{Gt/Gt}</i>
	<i>no losartan</i>	<i>no losartan</i>	<i>no losartan</i>	<i>no losartan</i>	<i>losartan</i>	<i>losartan</i>
	<i>16 weeks</i>	<i>16 weeks</i>	<i>24 weeks</i>	<i>24 weeks</i>	<i>24 weeks</i>	<i>24 weeks</i>
ALT (IU/L)	41.0±1.53	153±48.6	70.6±22.2	505±78.5	39±7.37	383±101
Triglycerides(mg/dl)	142±66.6	154±11.2	85.0±12.0	75.5±19.5	97.0±22.1	69.5±18.6
HDL (mg/dl)	65.6±8.03	95.2±13.4	66.7±2.01	123±25.4	72.8±14.7	147±18.3
Cholesterol (mg/dl)	81.0±13.8	107±10.7	94.4±2.18	141±35.0	84.7±16.9	169±20.6
Glucose (mg/dl)	146±10.8	177±17.1	182±9.03	186±31.5	152±25.2	208±10.9
Free fatty acids (mEq/L)	2.19±0.55	2.26±0.39	0.89±0.13	0.77±0.04	1.88±0.33	1.88±0.27

All results are expressed as averages ± standard error of the means. Values were considered statistically significant when the P-value was equal to or less than 0.05.

To provide an indicative value of liver damage, ALT levels were measured in all mouse groups both before and after losartan treatment. ALT (alanine transaminase) is an enzyme found in the liver and damage to the liver results in a release of ALT into the blood. Over the 8-week treatment period ALT levels increased more than threefold in 16-week old *Alms1^{Gt/Gt}* mutants (153 ± 48.6 IU/L, $n = 3$) when compared with 24-week old *Alms1^{Gt/Gt}* mice (505 ± 78.5 IU/L, $n = 4$) not treated with losartan (Figure 10). After treatment, 24-week old, losartan-treated *Alms1^{Gt/Gt}* mice (383 ± 101 IU/L, $n = 4$) ALT levels only increased by 60%, which was 24% less than untreated *Alms1^{Gt/Gt}* mutants at 24 weeks of age. A two-tailed statistical test of equal variance revealed this difference to be insignificant ($p = 0.326$). Prior to the start of treatment at 16 weeks of age, C57BL/6Ei wild type control mice ($n = 4$) average ALT levels were 41.0 ± 1.53 IU/L. Following the 8-week experimental period, C57BL/6Ei mice both treated (39 ± 7.37 IU/L, $n = 4$) and not treated (70.6 ± 22.2 IU/L, $n = 4$) with losartan did not have any significant change in ALT levels.

In order to track the phenotypic changes within the ALMS mouse model and those in response to losartan treatment, triglyceride levels in collected blood samples were analyzed (Figure 11). Results depicted a twofold decrease in triglyceride levels between 16-week old *Alms1^{Gt/Gt}* mice (154 ± 11.2 mg/dl, $n = 4$) and 24-week old *Alms1^{Gt/Gt}* mice (75.5 ± 19.5 mg/dl, $n = 2$) after 8 weeks. Following treatment with losartan, this decrease in triglyceride levels remained unchanged for *Alms1^{Gt/Gt}* mice at 24 weeks of age (69.5 ± 18.6 mg/dl, $n = 4$). A two-tailed statistical test of unequal variance revealed these changes to be insignificant ($p = 0.09$). Triglyceride levels of C57BL/6Ei wild type

control mice depicted a similar trend to those of *Alms1^{Gt/Gt}* mice. After 8 weeks, 16-week old C57BL/6Ei mice (142 ± 66.6 mg/dl, n= 3) triglyceride levels decreased approximately 40% for both 24-week old mice treated with losartan (69.5 ± 18.6 mg/dl, n =3) and those not treated with losartan (75.5 ± 19.5 mg/dl, n= 5). A two-tailed statistical test of unequal variance revealed these changes to be insignificant ($p = 0.481$).

HDL (high-density lipoprotein) levels were measured and analyzed in collected blood samples throughout the experimental period (Figure 12). After 8 weeks, HDL levels of 16-week old *Alms1^{Gt/Gt}* mice (95.2 ± 13.4 mg/dl, n= 4) increased by 16% when compared to 24-week old untreated *Alms1^{Gt/Gt}* mice (75.5 ± 19.5 mg/dl, n= 2). This increase was doubled to 35% for 24-week losartan treated *Alms1^{Gt/Gt}* mice (147 ± 18.3 mg/dl, n= 4) when compared with 16-week old mutants. A two-tailed statistical test of unequal variance revealed both changes to be insignificant ($p = 0.455$, and $p = 0.067$ respectively). C57BL/6Ei wild type control HDL levels at 16 weeks (65.6 ± 8.03 mg/dl, n= 3), 24 weeks untreated (66.7 ± 2.01 mg/dl, n= 5), and 24 weeks treated (72.8 ± 14.7 mg/dl, n= 3) were not altered over the course of the experiment.

Cholesterol levels were also measured to track *Alms1^{Gt/Gt}* phenotypic changes and to determine if the losartan treatment had an effect (Figure 13). After 8 weeks, cholesterol levels were 24% higher for 24-week old untreated *Alms1^{Gt/Gt}* mice (141 ± 35.0 mg/dl, n= 2) than 16-week old *Alms1^{Gt/Gt}* mice (107 ± 10.7 mg/dl, n= 4). A two-tailed statistical test of unequal variance revealed this change to be insignificant ($p = 0.498$). Similarly, cholesterol levels were demonstrated to be 37% higher for 24-week old *Alms1^{Gt/Gt}* mice (169 ± 20.6 mg/dl, n= 4) when compared to 16-week old mutants. A two-tailed statistical test of unequal variance revealed this change to be significant ($p = 0.049$). C57BL/6Ei

wild type control cholesterol levels at 16 weeks (81.0 ± 13.8 mg/dl, $n = 3$), 24 weeks untreated (94.4 ± 2.18 mg/dl, $n = 5$), and 24 weeks treated (84.7 ± 16.9 mg/dl, $n = 3$) had no significant changes over the course of the experiment.

Because both human patients and the ALMS mouse model exhibit signs of hyperglycemia, blood glucose levels were measured (Figure 14). Prior to the start of treatment, 16-week old *Alms1^{Gt/Gt}* mice glucose levels were 177 ± 17.1 mg/dl ($n = 4$). After 8 weeks, 24-week old *Alms1^{Gt/Gt}* mice (186 ± 31.5 mg/dl, $n = 2$) glucose levels were 5% higher than 16-week old mutants. Losartan treated 24-week old *Alms1^{Gt/Gt}* mice (208 ± 10.9 mg/dl, $n = 4$) glucose levels increased 15%, but did not significantly differ from 16-week and 24-week, untreated mice ($p = 0.830$ and $p = 0.179$, respectively). C57BL/6Ei controls at 16 weeks of age (146 ± 10.8 mg/dl, $n = 3$) and 24 weeks of age (152 ± 25.2 mg/dl, $n = 3$), treated, did not significantly change over the 8-week period. However, untreated C57BL/6Ei controls at 24 weeks of age (182 ± 9.03 mg/dl, $n = 5$) exhibited 17% higher average glucose levels. A two-tailed statistical test of unequal variance revealed this difference to be insignificant ($p = 0.057$).

Free Fatty Acid levels were measured to determine how they were affected by losartan treatment (Figure 15). Prior to the start of treatment, at 16 weeks of age, *Alms1^{Gt/Gt}* mice (2.26 ± 0.39 mEq/L, $n = 4$) free fatty acid levels were highest but decreased 66% at 24 weeks of age, untreated (0.77 ± 0.04 mEq/L, $n = 2$). A two-tailed statistical test of unequal variance revealed this difference to be significant ($p = 0.031$). twenty-four-week old losartan treated *Alms1^{Gt/Gt}* mice (1.88 ± 0.27 mEq/L, $n = 4$) free fatty acid levels decreased 17% when compared to 16-week old mutants. A two-tailed statistical test of unequal variance revealed this difference to be insignificant ($p = 0.464$). C57BL/6Ei

controls at 16 weeks of age (2.19 ± 0.55 mEq/L, n= 3) decreased 59% at 24 weeks of age, and untreated (0.89 ± 0.13 mEq/L, n= 5). A two-tailed statistical test of unequal variance revealed this difference to be insignificant ($p = 0.134$). 24-week old losartan treated *Alms1^{Gt/Gt}* mice (1.88 ± 0.27 mEq/L, n= 3) free fatty acid levels decreased 14% when compared to 16-week old mutants. A two-tailed statistical test of unequal variance revealed this difference to be insignificant ($p = 0.655$). The observed pattern of free fatty acid levels was the same for both *Alms1^{Gt/Gt}* and C57BL/6Ei mice.

Histological Analysis:

The extent of fibrosis and steatosis was assessed by histological evaluation of liver samples in both losartan treated *Alms1^{Gt/Gt}* and untreated groups (Figure 16). Liver sections of C57BL/6Ei wild type mice exhibited normal hepatocytes with no signs of fibrosis or hepatosteatois (Figure 16a). Normal hepatocytes were presented as clusters of cells with obvious nuclei, organized into 1-cell thick plates, separated by vascular channels. Liver sections of untreated *Alms1^{Gt/Gt}* mice at one year of age exhibited severe fibrosis as well as macro hepatosteatois (Figure 16b). Losartan treated mice at twenty-four weeks of age exhibited micro and macro hepatosteatois as well as fibrosis (Figure 16c, 16d). Micro hepatosteatois presented as white, clustered, bubble-like areas of the hepatocytes, caused by cellular retention of lipids. Macro hepatosteatois presented as larger, white, singular areas of clustered lipids. Fibrosis presented as darker stained areas within the vascular channels due to increases of collagen in these areas. Trials involving bezafibrate are still in progress.

Genomic Modifier Mapping:

Various phenotypic data was collected from *Alms1^{Gt/Gt}* on the C57BL/6Ei and Balb/cJ backgrounds (Table 2). ALT, glucose, and fibrosis levels were more severe in C57BL/6Ei backcrossed progeny while outer nuclear layers (outer layers of the retina) were fewer in Balb/cJ backcrossed progeny. Body weight, kidney weight, steatosis, insulin, cholesterol, and HDL were also higher in C57BL/6Ei backcrossed progeny, while triglycerides and free fatty acids were higher in Balb/cJ backcrossed progeny.

Table 2. Clinical Chemistry Analysis of F2- *Alms1^{Gt/Gt}* Backcross Progeny

<i>Parameters</i>	<i>(B6 X Balb)F1 X Balb Backcross</i>	<i>(B6 X Balb)F1 X B6 Backcross</i>
Body Weight (g)	47.2 ± 1.00	52.5 ± 1.48
Kidney Weight (g)	0.29 ± 0.01	0.35 ± 0.01
Liver Fibrosis	0.00 ± 0.00	0.89 ± 0.23
ONL Layers	6.72 ± 0.14	8.72 ± 0.14
Steatosis	0.96 ± 0.04	1.58 ± 0.15
Insulin (ng/ml)	110 ± 21.5	150 ± 24.1
Cholesterol (mg/dl)	137 ± 4.32	156 ± 10.7
HDL (mg/dl)	112 ± 2.97	131 ± 7.81
Triglycerides (mg/dl)	287 ± 15.3	136 ± 7.84
Free fatty acids (mEq/L)	1.79 ± 0.06	1.14 ± 0.04
ALT (IU/L)	103 ± 10.2	260 ± 46.3
Glucose (mg/dl)	199 ± 9.36	230 ± 17.9

Note: Numbers represent averages ± standard error of the means. Analysis was done for 32 Balb and 18 B6 backcross progeny. All mice were at 20 weeks of age. Liver fibrosis was judged on a scale of 0 to 3 with 3 the most severe. Steatosis was rated on a scale of 0 to 2 with 2 indicative of macrosteatosis and 1 of microsteatosis.

Utilization of Mapmanager QTX computer software resulted in identification of putative modifying loci in the (B6 X Balb)F1 X Balb and (B6 X Balb)F1 X B6 backcrosses (Table 3). Reported putative modifying loci were limited by p-values <0.001 and <0.01. Genotype markers were omitted from analysis for incomplete data. However,

genotypes were inferred from flanking markers when the genotypes agreed; these markers were not treated as missing data (Manly *et al.*, 1999). A chromosome map showing relative locations of the genomic modifiers is shown in Figure 17.

Table 3. Positions and phenotypic attributes of QTLs for HDL, ALT, insulin, body weight, free fatty acids, fibrosis, and glucose detected in F2- *Alms1^{Gt/Gt}* Backcrosses

Backcross	Phenotype	Chr	Position (cM)	Marker	p-value	Symbol
(B6 X Balb)F1 X B6	HDL	5	19.7	<i>D5Mit233</i>	0.000048	Ahd1B6
	ALT	4	66.7	<i>D4Mit251</i>	0.00479	Alt1B6
		4	30.6	<i>D4Mit178</i>	0.00173	Alt2B6
	Insulin	8	42.6	<i>D8Mit240</i>	0.00134	AinsB6
	Body weight	4	30.6	<i>D4Mit178</i>	0.00727	AbwB6
	Free fatty acids	13	47	<i>D13Mit262</i>	0.00659	AffaB6
	Fibrosis	19	1.1	<i>D19Mit109</i>	0.00112	AfB6
(B6 X Balb)F1 X Balb	Retinal Degeneration	2	72.1	<i>D2Mit493</i>	0.00003	ArdB
	Body weight	1	61.2	<i>D1Mit135</i>	0.00378	Abw1B
		2	68.9	<i>D2Mit282</i>	0.00557	Abw2B
	Insulin	15	29.5	<i>D15Mit156</i>	0.00342	AinsB
	ALT	3	57.9	<i>D3Mit44</i>	0.00518	AltB
	Glucose	8	12	<i>D8Mit4</i>	0.00966	Ag1uB

Note: Position (cM) of marker locus (Mouse Genome Database) with peak lod score.

Discussion

Alström Syndrome (ALMS) is a clinically progressive genetic disorder characterized by vision and hearing loss, obesity, steatosis, fibrosis, insulin resistance and its associated complications. Currently, there are few treatments for these phenotypes. The experiment described herein sought to address the hepatosteatosis and fibrosis phenotypes using the previously created *Alms1^{Gt/Gt}* mouse model.

In this study, losartan treatment of *Alms1^{Gt/Gt}* mice, beginning at 16 weeks of age and continuing for 8 weeks, had little effect on phenotypic improvement. The increase in ALT levels (which are indicative of liver damage) observed over the 8-week period for both treated and untreated *Alms1^{Gt/Gt}* mice was likely a result of the condition worsening over time. The small decrease in ALT levels within treated *Alms1^{Gt/Gt}* mice when compared to untreated *Alms1^{Gt/Gt}* mice may have been a result of the losartan treatment. However, because the difference is not significant it cannot be concluded that the difference was a result of the treatment. Triglycerides were observed to be the highest in 16-week old mice of all groups and to have decreased over the 8-week period for both treated and untreated wild type and *Alms1^{Gt/Gt}* mice. Because this decrease was seen in both *Alms1^{Gt/Gt}* and wild type mice, as well as treated and untreated mice, it can be assumed that it had nothing to do with the progression of the *Alms1^{Gt/Gt}* phenotype or losartan. Possible reasons for this change may be found within the mouse lifecycle or it may be due to the small number of mice in this study. Further research with a greater number of mice would provide more information. HDL and cholesterol levels were observed to have the same pattern of change over the 8-week period for *Alms1^{Gt/Gt}* mice. This was not unexpected because of the relationship between HDL and cholesterol. HDL

is used to transport the hydrophobic molecule cholesterol throughout the aqueous environment of the body (Kwiterovich, 2000). Therefore, any increase in cholesterol would likely trigger an increase in HDL synthesis for the purpose of transport. It was interesting to note that both HDL and cholesterol levels in *Alms1^{Gt/Gt}* mice increased over the 8-week period and were highest in those treated with losartan. Previous studies have shown losartan to slightly increase HDL and cholesterol in human patients with hypertension and type 2 diabetes (Lozano *et al.*, 2001). In addition, other studies have found that increases in cholesterol suppress cellular TGF- β responsiveness in vascular cells (Chen *et al.*, 2007). This may suggest that cholesterol has some protective benefits against TGF- β and perhaps fibrosis in *Alms1^{Gt/Gt}* mice and human ALMS patients. Glucose levels were not observed to have changed much as a result of losartan treatment. This was not unexpected as there is no data suggesting losartan has any affect on glucose levels. Glucose levels of treated 24-week old *Alms1^{Gt/Gt}* mice were observed to be slightly higher than other *Alms1^{Gt/Gt}* mice and wild types. Because all measured glucose levels are approximately the same, it is likely that any differences among groups are the result of low numbers of mice in the study. Observed free fatty acid levels did not differ between *Alms1^{Gt/Gt}* mice and their wild type counterparts. This suggests that the genotype (either *Alms1^{Gt/Gt}* or wild type) did not influence free fatty acid levels. Free fatty acid levels did differ between experimental groups. Free fatty acid levels were highest for 16-week old mice and lowest for 24-week old untreated mice. This suggests that losartan had an effect on free fatty acid levels. Previous studies have shown increases in free fatty acids as a result of losartan treatment (Fellmann *et al.*, 2009). Further experiments with more mice would be necessary to validate these results. Tissue

analysis of losartan treated *Alms1^{Gt/Gt}* mice was indistinguishable from untreated *Alms1^{Gt/Gt}* mice. This result was not unexpected due to the observed high ALT levels, which are correlated to liver damage, and did not significantly differ between treated and untreated *Alms1^{Gt/Gt}* mice. Longer treatment periods may see an improvement in histological samples.

Bezafibrate is a drug that has been shown to inhibit free fatty acid composition and promote beta-oxidation. Bezafibrate treatment of *Alms1^{Gt/Gt}* mice is in progress. Other fatty acid oxidation enhancers such as Wy14,643 have been shown to reduce microsteatotic and macrosteatotic change in *foz/foz* mice, which are phenotypically similar to *Alms1^{Gt/Gt}* mice (Larter *et al.*, 2012). In these studies, steatotic improvements were seen following 10 days of treatment. In the present study, treatment with bezafibrate will continue in conjunction with losartan.

In most cases, human diseases are not treated until the phenotypic symptoms of the disease emerge. By administering losartan at 16 weeks of age, and after the appearance of the ALMS phenotype, treatment was intended to simulate treatment in human ALMS patients. Previous studies have identified a correlation between increased transforming growth factor (TGF- β) and increased procollagen I mRNA levels (Kagami *et al.*, 1994). TGF- β is known to influence other biochemical pathways that regulate cell division, heart disease, diabetes and more. Losartan, a selective angiotensin II type I receptor antagonist, has been shown to reduce both TGF- β and procollagen I mRNA thus decreasing hepatic fibrosis in certain human and animal models (Yoshiji *et al.*, 2001). In a preliminary study with *Alms1^{Gt/Gt}* mice, losartan was administered to mice beginning at approximately 3 weeks of age, before development of the ALMS phenotype, and

treatment continued until 9 months of age (Collin *et al.*, unpublished). Analysis of these data suggested that losartan significantly lowered ALT levels in treated *Alms1^{Gt/Gt}* mice compared to untreated *Alms1^{Gt/Gt}* mice. In the present experiments, results suggested losartan had little effect in relieving the fibrosis observed in the *Alms1^{Gt/Gt}* mouse model. Based on the success of previous studies, it is possible that a treatment period of 8 weeks is not enough time to observe a significant change. Studies have shown that losartan treatment over a 6-month period significantly lowered pulmonary fibrosis (Habashi *et al.*, 2006). In previous studies of Marfan Syndrome, losartan was administered prenatally and continued until 10 months of age (Habashi *et al.*, 2006). Fibrosis was diminished in the tissues of these treated mice. These data suggest prolonged treatment with losartan exceeding 2 months of treatment may result in decreased fibrosis in liver samples. Currently, there is no singular treatment for ALMS. This study may help identify alternative and less harmful treatments for the multiple phenotypes associated with ALMS. By treating severe phenotypes—fibrosis and steatosis—the onset of systemic fibrosis observed in ALMS patients may be prevented or delayed. Moreover, these treatments may extend to other diseases such as NASH where fibrosis and steatosis are prevalent.

Diagnosis and treatment of ALMS are difficult because of the differences in the onset, progression and absence or presence of the associated phenotypes (Marshall, 2005). These phenotypic differences among patients may be due to genetic background modifiers as well as the influence of environmental factors. Utilization of the *Alms1^{Gt/Gt}* mouse model to study ALMS enables a degree of environmental and genetic control unreachable in human patients. Through this control, possible environmental effects can

be minimized and genomic modifiers identified. Several genomic modifiers were identified with significance $p < 0.05$. With the backcross to C57BL/6Ei, significant modifying loci were HDL and suggestive loci were ALT, insulin, body weight, free fatty acids, and fibrosis. With the backcross to Balb/cJ, significant modifying loci were retinal degeneration and suggestive loci were body weight, glucose, ALT and insulin. Previous studies have proposed a modifier for retinal degeneration in the *tub/tub* mouse model on chromosome 2 (Andersen *et al.*, 2003). *Alms1^{Gt/Gt}* and *tub/tub* mice are similar phenotypically—sharing adult-onset obesity and progressive retinal and cochlear degeneration (Collin *et al.*, 2002). The QTL on chromosome 2 that modifies retinal degeneration in the *tub/tub* mouse lies between chromosomal positions 34.4 and 77.2 cM. The QTL on chromosome 2 that modifies retinal degeneration in the *Alms1^{Gt/Gt}* mouse model is located around 72.1cM. These data may indicate that the modifier for retinal degeneration may be the same in both *tub/tub* and *Alms1^{Gt/Gt}* mice. A candidate gene modifier for *Alms1* and *Tub* may be *BBS6/MKKS*, which lies within chromosomal positions 34.4 and 77.2 cM. Previous studies have shown that patients with mutations in the *BBS6* gene have clinical features similar to ALMS and both proteins localize to the centrosome (Marshall, 2005). Identification of significant modifying loci may provide more information as to the progression and early detection of phenotypes associated with ALMS. This would require further study to positively associate genotype with phenotype.

Although losartan was shown to reduce the levels of some phenotypic traits associated with the *Alms1^{Gt/Gt}* mouse model, it was also shown to increase others. While these changes were generally insignificant, other studies have shown longer treatment

periods starting at an earlier time would improve the degree of changes observed as a result of losartan treatment. Several genomic loci that may influence phenotypic observations within *Alms1*^{Gt/Gt} mice were identified. These findings, when compiled with those from future experiments, may offer ways to identify ALMS progression in human patients.

Figures

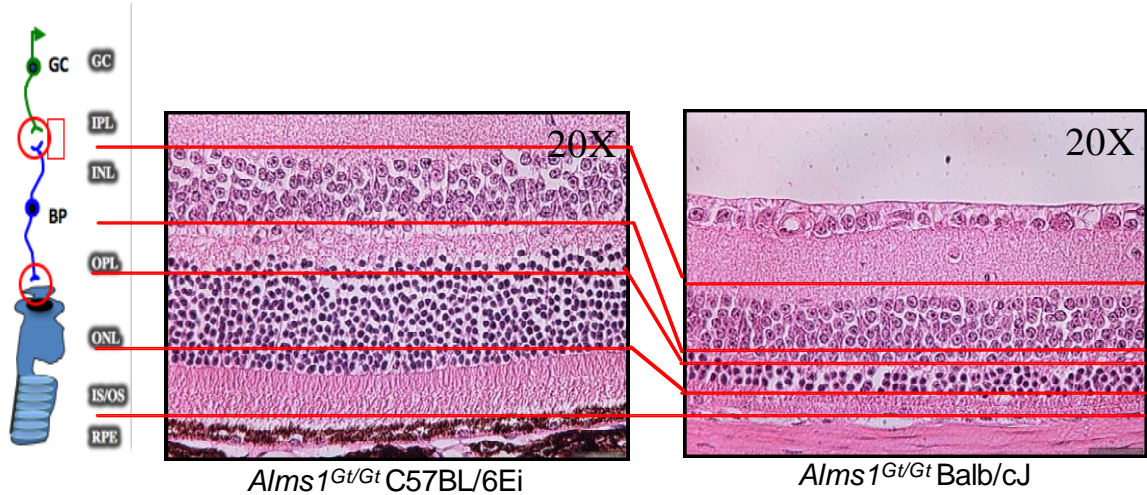


Figure 1: Background Retinal Degeneration. Retinal degeneration differed between *Alms1^{Gt/Gt}* C57BL/6Ei and Balb/cJ mice of 24 weeks of age. Degeneration is more severe in Balb/cJ mice (right), which is represented by fewer outer nuclear layers (ONL), and thinner outer plexiform (OPL) and rods and cones (IS/OS). Both the ganglion cell layer (GC) and the inner plexiform layer (IPL) are not shown in the C57BL/6Ei retina image (left). Retinal pigment epithelium (RPE) appeared different due to the tendency of black eyes in C57BL/6Ei mice and red eyes in Balb/cJ mice. Images courtesy of Gayle Collin.

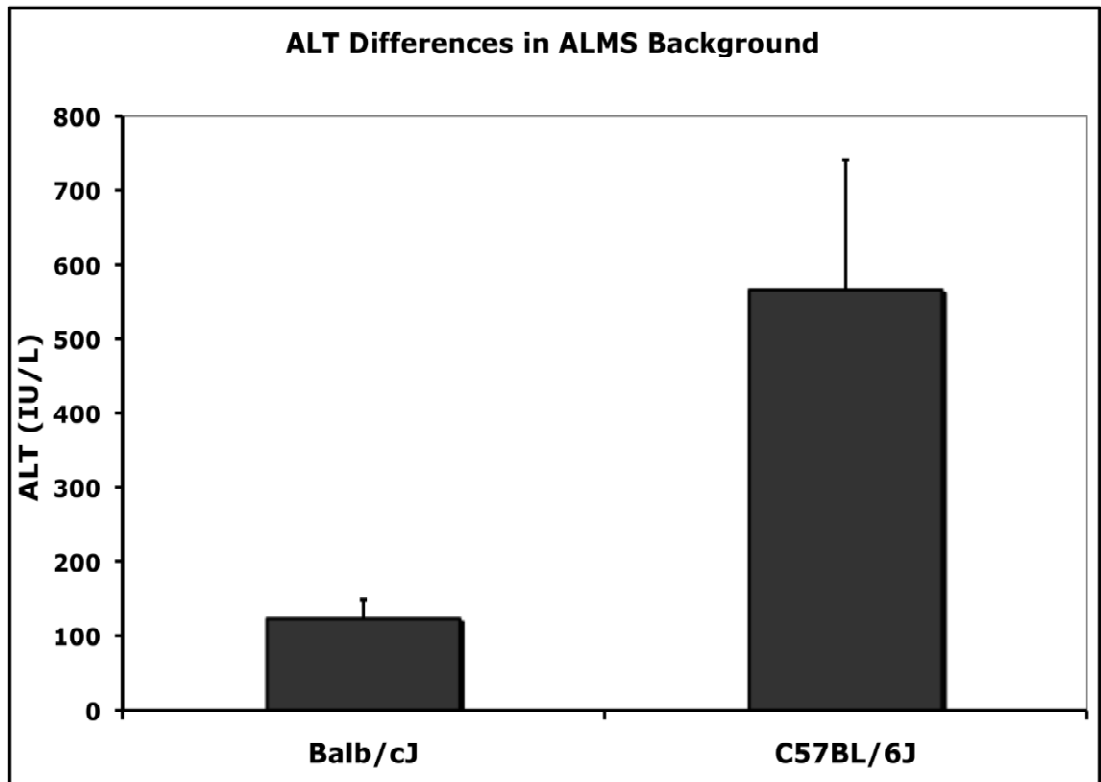


Figure 2: Background ALT Levels. ALT levels differed between *Alms1^{Gt/Gt}* C57BL/6Ei and Balb/cJ mice of the same age. C57BL/6J mice ALT levels were approximately higher than those of Balb/cJ mice. Image courtesy of Gayle Collin.

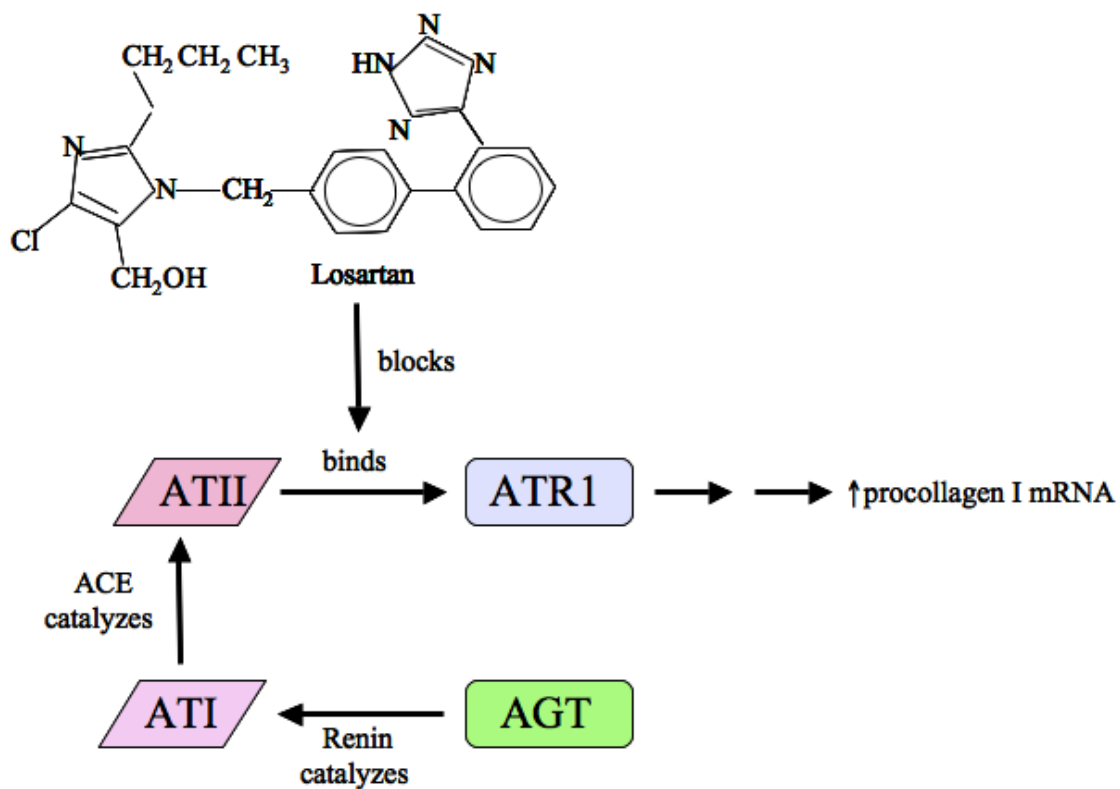


Figure 3: Action of Losartan. The mechanism of action of losartan within the renin-angiotensin system (RAS). Angiotensinogen (AGT) produced by the liver is converted to angiotensin-I (ATI) by rennin, which is produced in the kidneys (Yoshiji *et al.*, 2001). Angiotensin-converting enzyme (ACE) then cleaves ATI to angiotensin-II (ATII). ATII then binds to angiotensin II type 1 receptor (ATR1), which causes a cascading effect that induces an increase in the translation of procollagen I mRNA and an increase in fibrotic tissue. Losartan blocks the binding of ATII to ATR1, dampening the cascading effect that results in an increase of fibrotic tissue. Figure adapted from Yoshiji *et al.*, 2001.

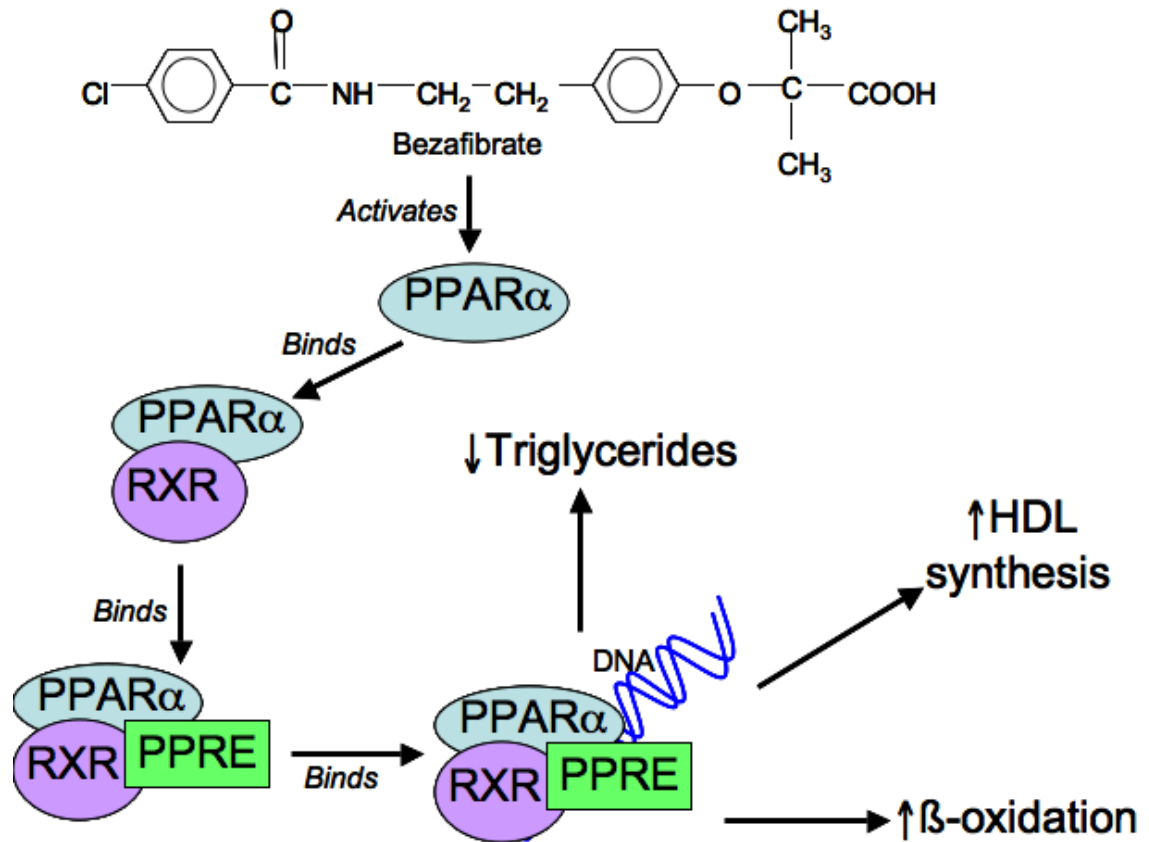


Figure 4: Action of Bezafibrate. Bezafibrate binds with and activates PPAR α (peroxisome proliferators-activated receptor- α) causing PPAR α to bind and form a heterodimer with RXR (retinoid X receptor). Together both PPAR α and RXR bind PPRE (peroxisome proliferator response elements) which influences gene expression. Transcription of target genes causes an increase in HDL synthesis and β -oxidation and causes a decrease in triglycerides (Goldenberg *et al.*, 2008). Figure adapted from Goldenberg *et al.*, 2008.

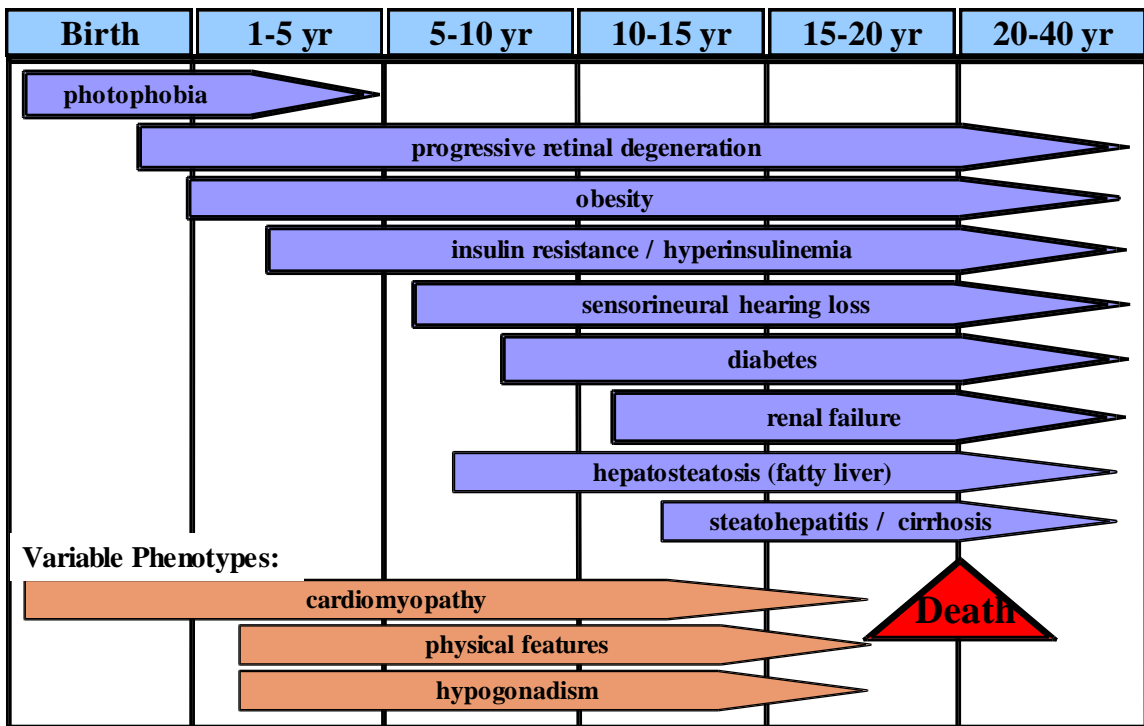


Figure 5: ALMS Phenotypes, Progression, and Approximated Age of Appearance. Phenotypes on blue arrows are known as cardinal features of ALMS, and are present in most patients (Marshall *et al.*, 2005). Variable phenotypes include, but are not limited to, those listed on orange arrows (Marshall *et al.*, 2007). Image adapted from Jan Marshall.

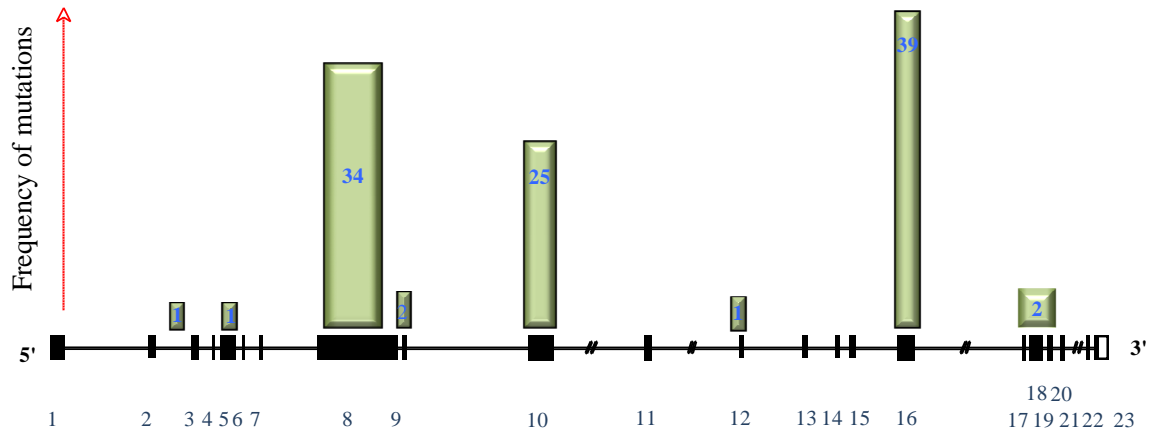


Figure 6: Frequency of Exon Mutations in Human *ALMS1*. Genotype examinations of 105 ALMS patients demonstrated the most frequent mutations were found on exons 8, 16, and 10 on chromosome 2, position 13. Mutations have been characterized as nonsense or frameshift mutations causing a prematurely terminated protein (Hearn *et al.*, 2002). Image courtesy of Gayle Collin.

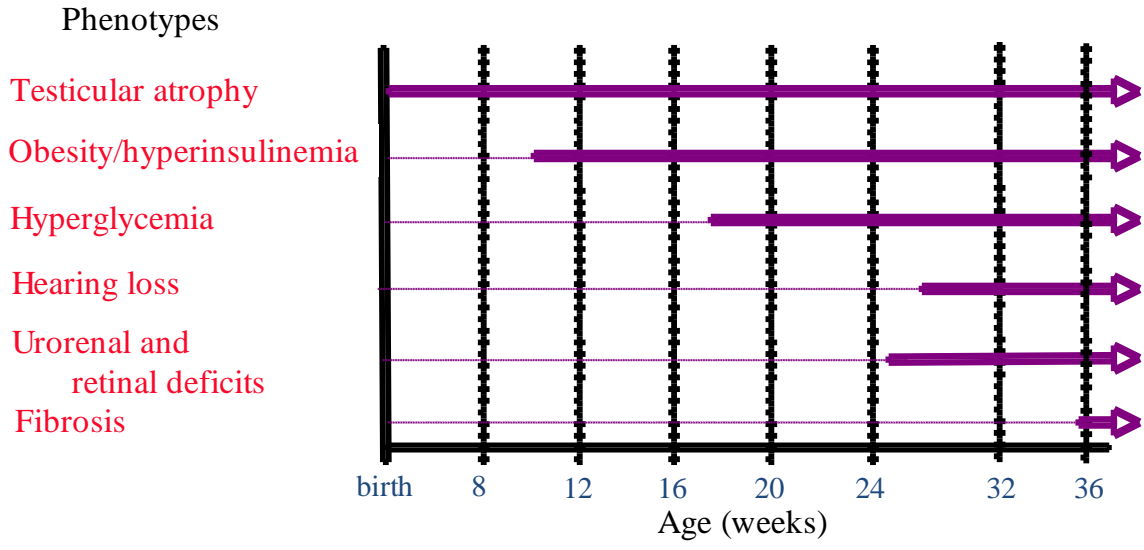


Figure 7: *ALMS1* Mutation Phenotypes and Progression in Mice. Many phenotypes observed in human ALMS patients were also seen in the *Alms1^{Gt/Gt}* mouse model. Figure adapted from Gayle Collin.

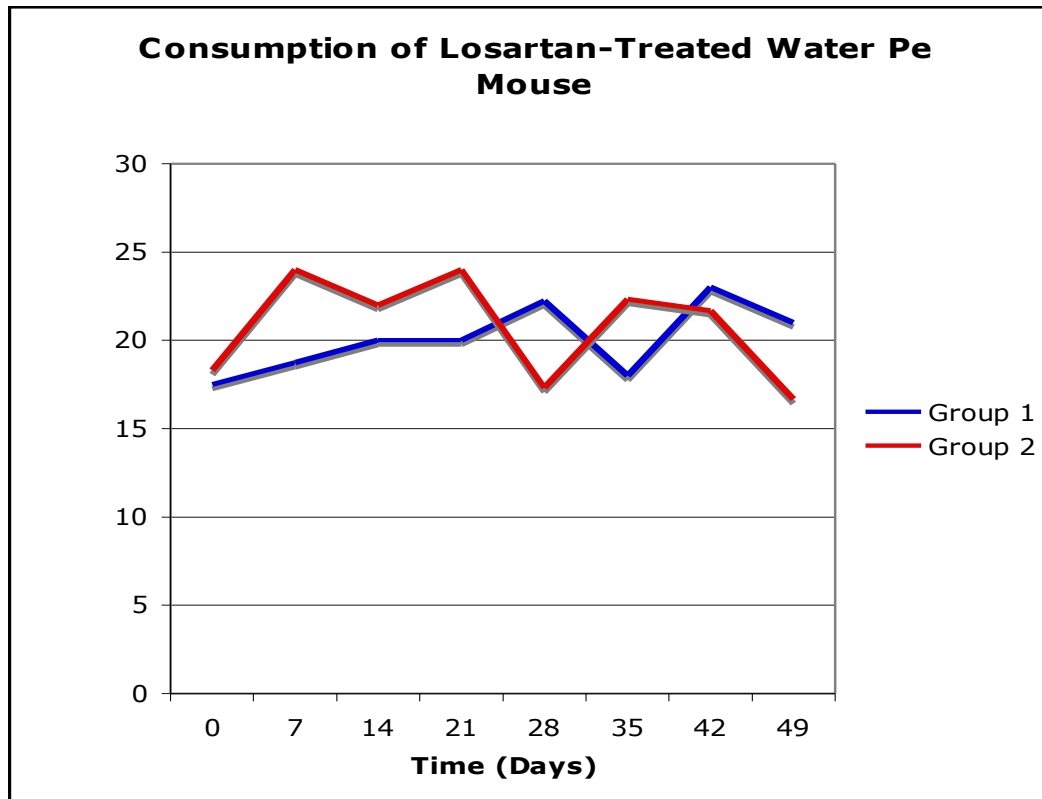


Figure 8: Losartan-Treated Water Consumption per Week. Losartan treated water consumption was proportionally similar for group 1 and group 2. Group 1 consisted of four mice, three of which were $Alms1^{Gt/Gt}$ and one was C57BL/6Ei wild type. Group 2 consisted of three mice, one of which was $Alms1^{Gt/Gt}$ and two were $Alms1^{Gt/+}$. Water consumption was measured every seven days. Data were then averaged and normalized according to number of $Alms1^{Gt/Gt}$ mutants in each group.

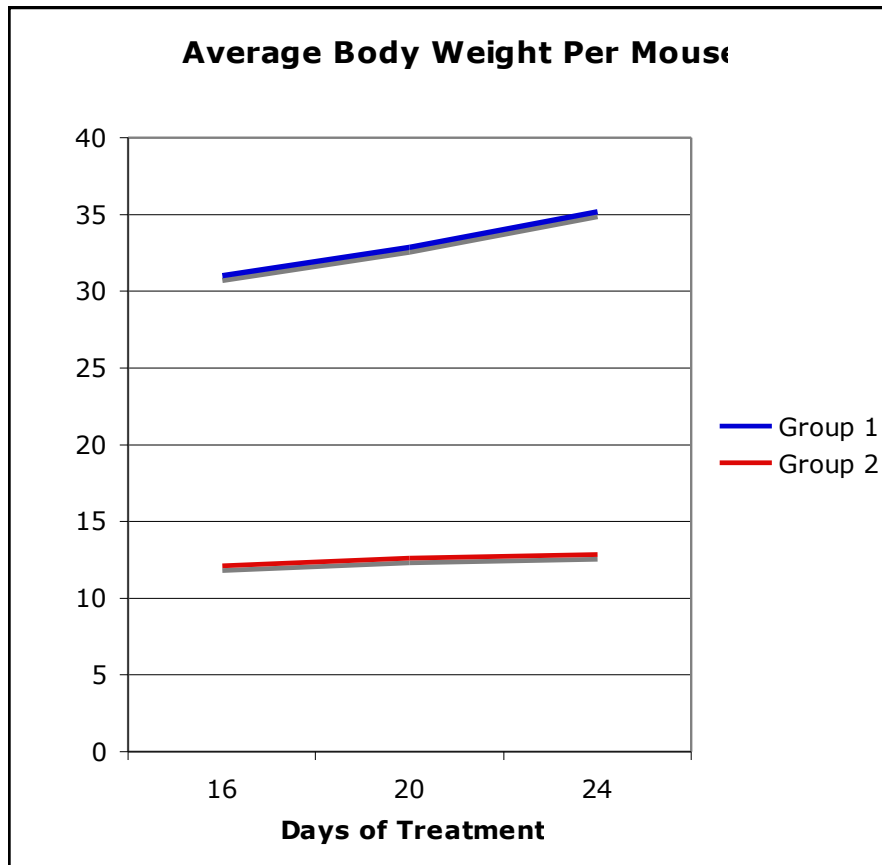


Figure 9: Body Weight. Average body weights of Group 1 were proportionally greater than Group 2. Trial 1 consisted of four mice, three of which were $Alms1^{Gt/Gt}$ and one was C57BL/6J wild type. Trial 2 consisted of three mice, one of which was $Alms1^{Gt/Gt}$ and two were $Alms1^{Gt/+}$. Body weight was measured at 16 weeks of age, 20 weeks of age, and 24 weeks of age. Data were then averaged and normalized according to number of $Alms1^{Gt/Gt}$ mutants in each group. Previous data suggests the volume of water consumed is influenced by body weight.

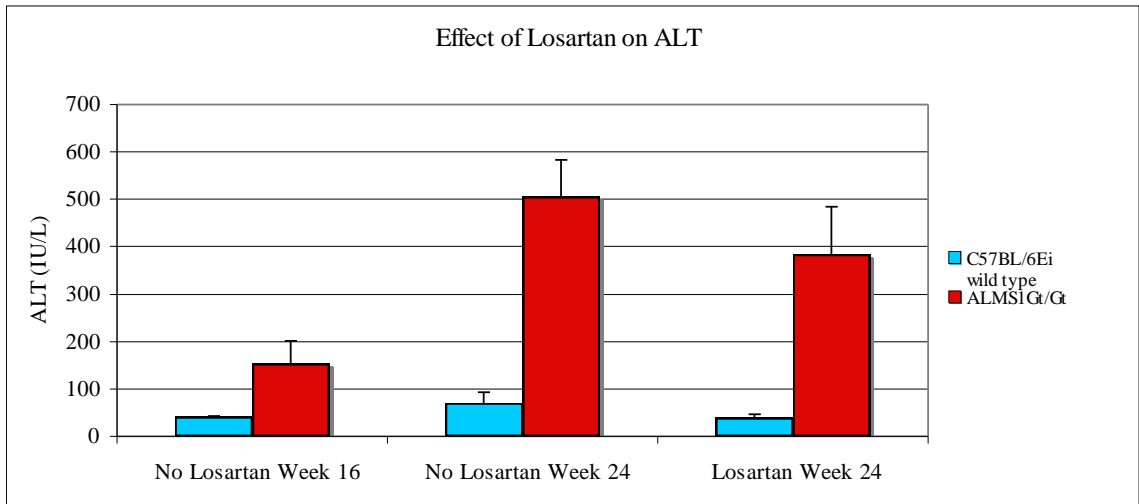


Figure 10: ALT Levels Before and After Losartan Treatment. Over the 8 week experimental period ALT levels increased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average ALT levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average ALT levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 4; n = 4). The increase in ALT levels for 24-week-old *Alms1^{Gt/Gt}* mice after losartan treatment was less in comparison to untreated *Alms1^{Gt/Gt}* mice of the same age. A two-tailed statistical test of equal variance revealed this difference to be insignificant (p = 0.326). *Alms1^{Gt/Gt}* mice had higher ALT levels than their wild type controls. Wild type controls varied little between groups. Error was calculated as standard error of the means.

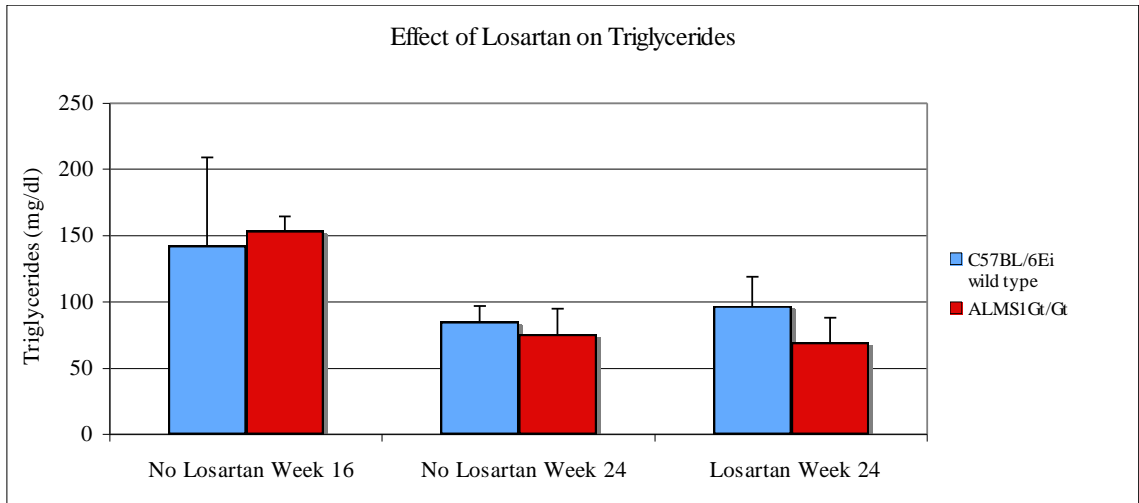


Figure 11: Triglycerides Before and After Losartan Treatment. Over the 8-week experimental period, triglyceride levels decreased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average triglyceride levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average triglyceride levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 2; n = 4). Triglyceride levels decreased in 24-week-old *ALMS1^{Gt/Gt}* mice after losartan treatment in comparison to untreated *Alms1^{Gt/Gt}* mice of the same age. A two-tailed statistical test of unequal variance demonstrated this difference to be insignificant (p = 0.839). Triglyceride levels decreased by approximately 40% in both *Alms1^{Gt/Gt}* and wild type mice after 24 weeks with and without treatment. A two-tailed statistical test of unequal variance showed the difference between untreated controls at 16 and 24 weeks to be insignificant (p = 0.481). Error was calculated as standard error of the means.

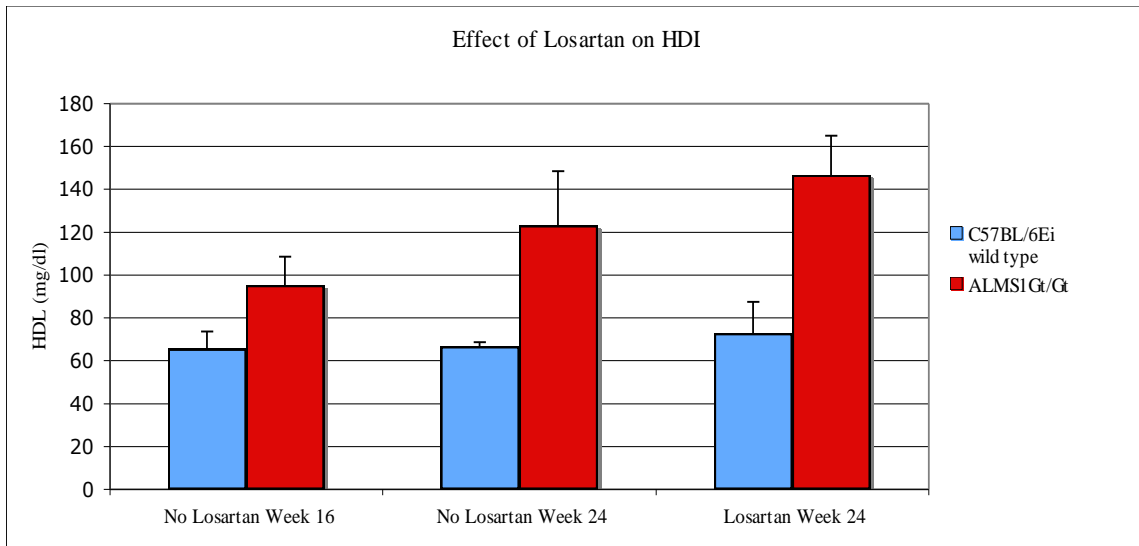


Figure 12: HDL Before and After Losartan Treatment. Over the 8-week experimental period HDL levels increased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average HDL levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average HDL levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 2; n = 4). After 24 weeks-of-age, mice without losartan treatment had 35% higher HDL levels than those at 16 weeks of age. HDL levels were approximately 15% higher in 24-week-old *Alms1^{Gt/Gt}* mice after losartan treatment in comparison to untreated *Alms1^{Gt/Gt}* controls of the same age. A two-tailed statistical test of unequal variance showed this difference to be insignificant (p = 0.525). *Alms1^{Gt/Gt}* mice had higher HDL levels than their wild type controls. Wild type control HDL levels were not affected over the 8-week experimental period. Error was calculated as standard error of the means.

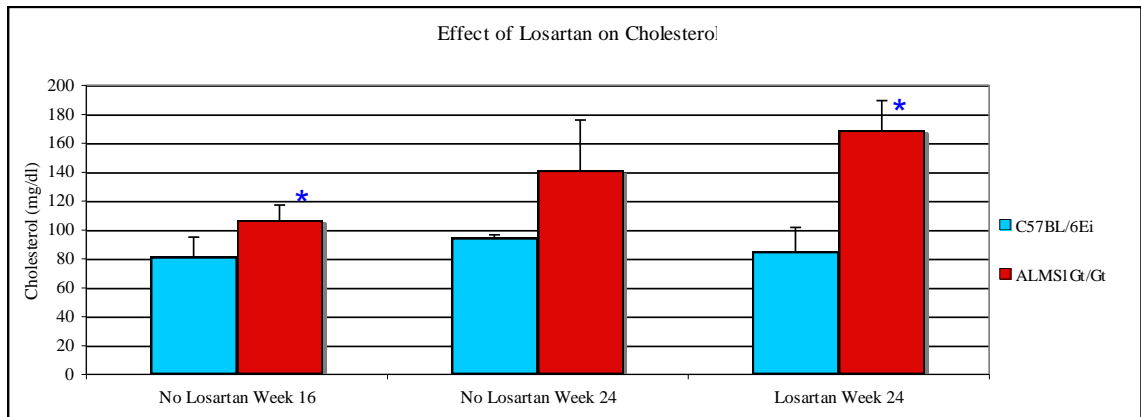


Figure 13: Cholesterol Before and After Losartan Treatment. Over the 8-week experimental period cholesterol levels increased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average cholesterol levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average cholesterol levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 2; n = 4). Cholesterol levels increased in 24-week-old *ALMS1^{Gt/Gt}* mice after losartan treatment in comparison to untreated *Alms1^{Gt/Gt}* controls of the same age. A two-tailed statistical test of unequal variance revealed this difference to be insignificant (p = 0.574). *Alms1^{Gt/Gt}* mice had higher cholesterol levels than their wild type controls. Wild type control cholesterol levels were not affected over the 8-week experimental period. Error was calculated as standard error of the means.

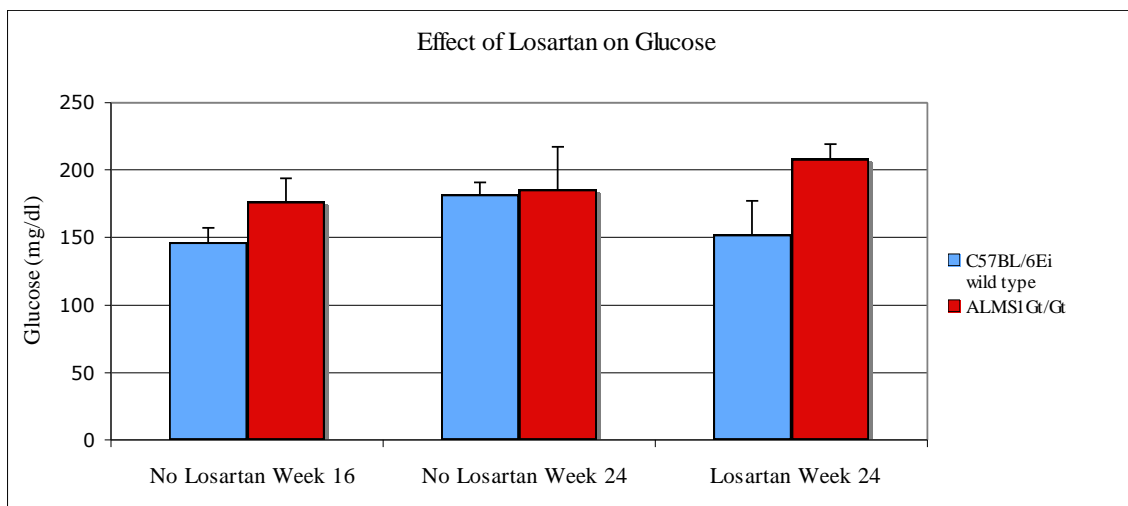


Figure 14: Glucose Before and After Losartan Treatment. Over the 8-week experimental period glucose levels increased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average glucose levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average glucose levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 2; n = 4). Glucose levels increased in 24-week-old *Alms1^{Gt/Gt}* mice after losartan treatment in comparison to untreated *Alms1^{Gt/Gt}* controls of the same age. A two-tailed statistical test of unequal variance demonstrated this difference to be insignificant (p = 0.603). *Alms1^{Gt/Gt}* mice had higher glucose levels than their wild type controls. Wild type control glucose levels were highest for untreated mice at 24 weeks of age during the 8-week experimental period. A two-tailed statistical test of unequal variance revealed the difference between untreated controls at 16 and 24 weeks to be insignificant (p = 0.057). Error was calculated as standard error of the means.

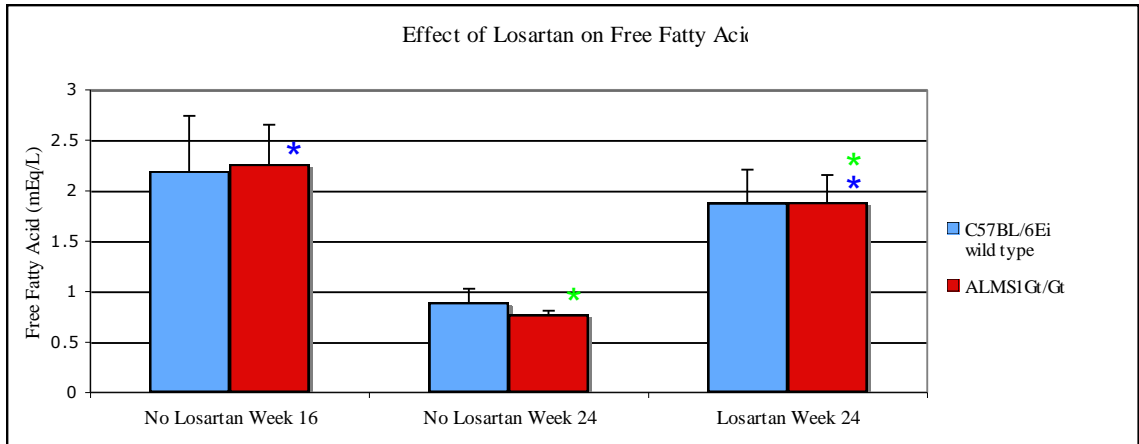


Figure 15: Free Fatty Acids Before and After Losartan Treatment. Over the 8-week experimental period free fatty acid levels decreased in both untreated and treated *Alms1^{Gt/Gt}* mice at 24 weeks of age. Average free fatty acid levels are shown for untreated wild type mice at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 3; n = 5; n = 3). Average free fatty acid levels are shown for untreated *Alms1^{Gt/Gt}* mutants at 16 weeks of age, 24 weeks of age, and treated at 24 weeks of age (n = 4; n = 2; n = 4). A two-tailed statistical test of unequal variance demonstrated the difference of free fatty acid levels between *Alms1^{Gt/Gt}* mice at 16 and 24 weeks of age to be significant (p = 0.031). Free fatty acid levels increased in 24-week-old *Alms1^{Gt/Gt}* mice after losartan treatment in comparison to untreated *Alms1^{Gt/Gt}* controls of the same age. A two-tailed statistical test of unequal variance revealed this difference to be significant (p = 0.026). *Alms1^{Gt/Gt}* mice had higher free fatty acid levels than their wild type controls. Wild type control free fatty acid levels decreased during the 8-week experimental period. A two-tailed statistical test of unequal variance revealed the difference of free fatty acid levels between wild type mice at 16 and 24 weeks to be insignificant. Error was calculated as standard error of the means.

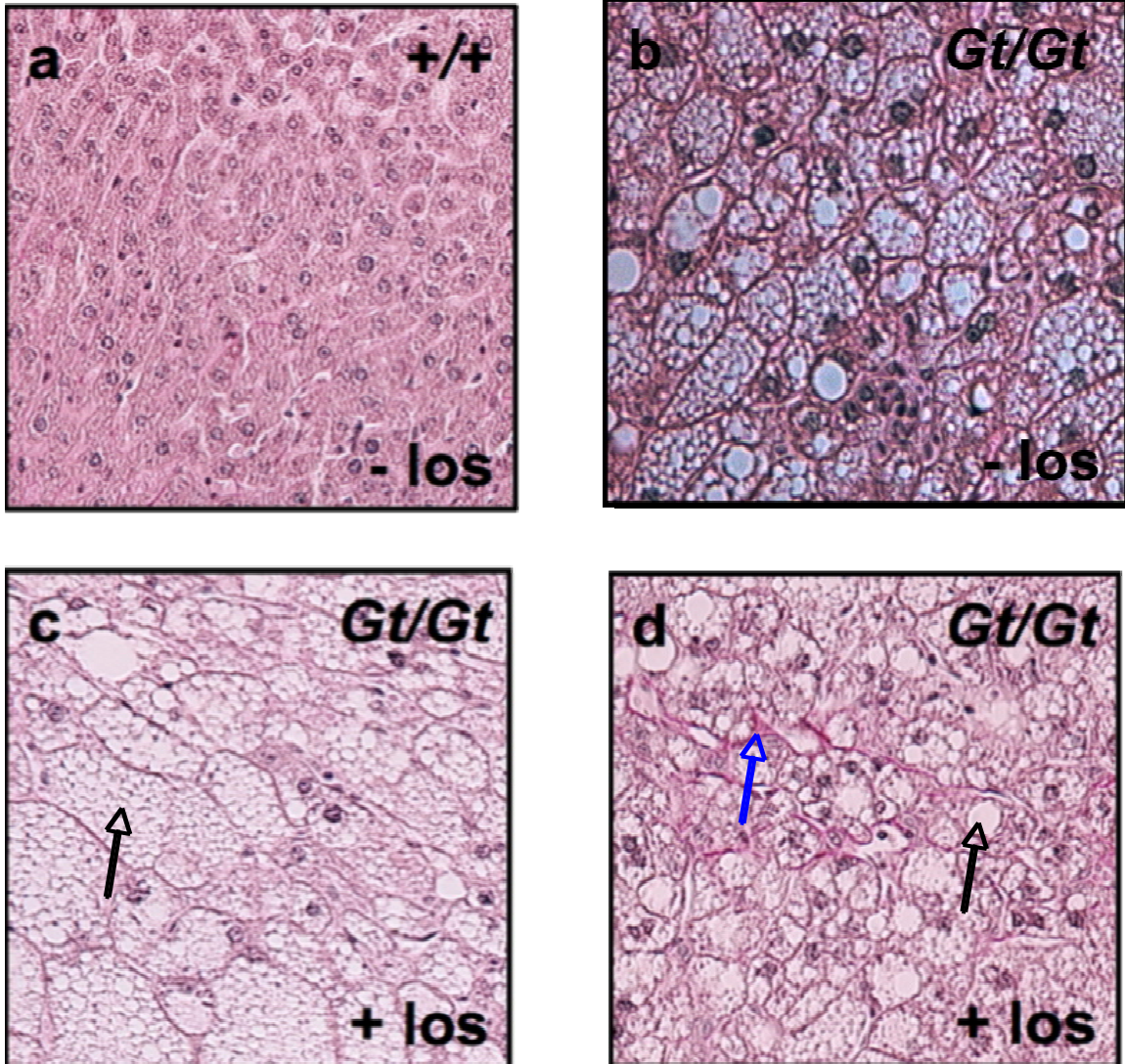


Figure 16: Liver Histological Analysis for Losartan Study. Histological analysis of treated *Alms1^{Gt/Gt}* mice at 24 weeks of age exhibited signs of fibrosis. Images were taken of a wild type mouse not treated with losartan at 24 weeks of age (a), an *Alms1^{Gt/Gt}* mouse not treated with losartan at 24 weeks of age (b), and *Alms1^{Gt/Gt}* mice treated with losartan at 24 weeks of age (c and d). Untreated, 24-week-old mouse (a), exhibited no signs of steatosis or fibrosis. Sections b, c, and d all exhibited signs of fibrosis and both micro and macro steatosis. Fibrosis was most obvious in section d where it was seen as darker red spacing between hepatocytes indicated by the blue arrow. Microsteatosis was seen in small white spaces ballooning the hepatocytes as seen in section c, indicated by the black arrow. Macrosteatosis was seen as larger white spaces as seen in section d, indicated by the black arrow.

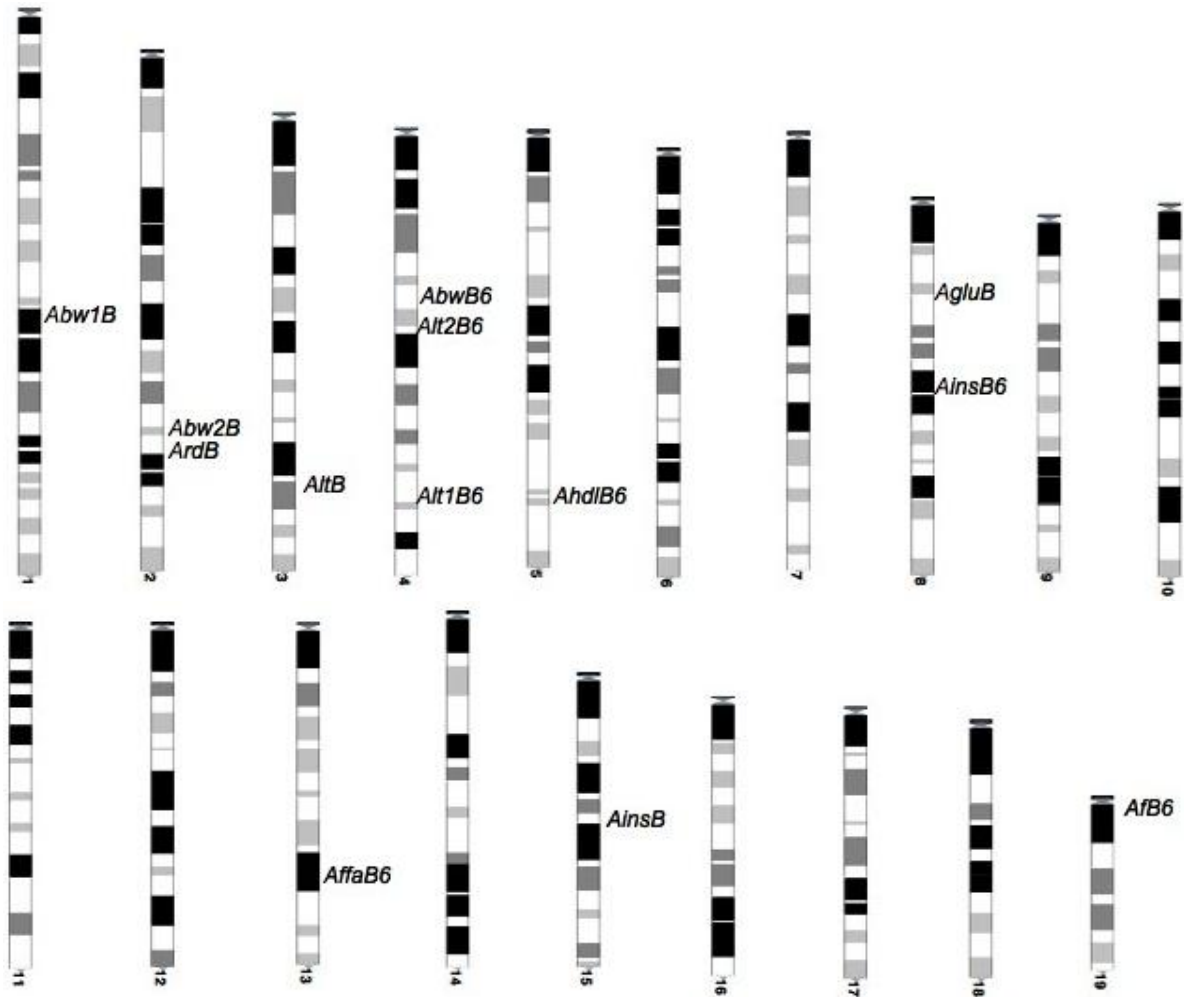


Figure 17: Relative Positions of Genomic Modifiers on Associated Chromosomes. Mouse karyotype with the approximated locations of statistically significant modifier loci designated by symbol names. Symbol names were derived by abbreviation of phenotype and backcross. Phenotypes differed based on backcross. (B6 X Balb)F1 X B6 backcrosses exhibited fibrosis and (B6 X Balb)F1 X Balb backcrosses exhibited retinal degeneration. Statistical analysis performed by Mapmanager found AhdIB6 on chromosome 5 and ArdB on chromosome 2 to be most significant ($p < 0.00005$).

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