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Sabrina Dallas April 12th, 2022

A characterization of prenatal metabolites in GALT-null rat fetuses

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Abstract

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Classic galactosemia (CG) is a rare genetic disorder characterized by a profound deficiency of galactose-1-phosphate uridylyltransferase (GALT). Accumulation of metabolites involved in the Leloir Pathway, of which GALT is an integral enzyme, starting during gestation and continuing through life is thought to be responsible for the complications observed, which include developmental delays, motor control issues, speech and cognitive disorders, and premature ovarian insufficiency. In the GALT-null rat model used in the Fridovich-Keil Lab to model CG, excess pre-weaning neonatal demise was observed in GALT-null pups from GALT-null by GALT-null crosses only. This study aimed to gain a better understanding of changes in Leloir Pathway metabolites through gestation and determine if there is a prenatal cause for early neonatal demise of GALT-null pups with two GALT-null parents. Galactitol, galactose, and galactose-1-phosphate (Gal-1P) levels were measured in livers, brains, and amniotic fluid at gestational days (GDs) 15, 17, 19, and 21 in order to address these goals. Results indicated that increased metabolite accumulation was present in GALT-null rat fetuses compared to heterozygous fetuses by GD 15, and that this metabolite accumulation increased through gestation. This ultimately suggests that treatments designed for CG must either be administered prior to this point in gestation or correct previous damage as well as preventing future damage. Results also indicated that increased proportions of co-gestating heterozygous siblings had a negative correlation with overall metabolite levels, suggesting some sort of cross-correction happening between fetuses. This suggests that prenatal accumulation of metabolites in GALT-null fetuses from crosses involving no heterozygotes may be one cause of excess neonatal demise observed in these rats.

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CHAPTER 1: BACKGROUND AND INTRODUCTION

Description of Classic Galactosemia

Classic galactosemia (CG) is an autosomal recessive disorder characterized by profound deficiency of galactose-1-phosphate uridylyltransferase (GALT) (1). The incidence of CG in the United States is approximately 1:50,000 (2). In populations with newborn screening (NBS) for CG, most affected babies are detected by NBS on the basis of low to undetectable GALT activity and/or elevated total galactose in a dried blood spot. A diagnosis of CG is subsequently confirmed by a more quantitative analysis of GALT activity and elevated galactose-1-phosphate (Gal-1P) levels in a fresh samples of red blood cells (1).

The early signs of CG in neonates include jaundice, poor feeding, cataracts, lethargy, vomiting, and failure to thrive (1, 3). Implementing a galactose-restricted diet within the first few days of life generally prevents the liver failure and sepsis that can lead to neonatal death. However, developmental and other complications present as the child grows despite dietary galactose restriction. The complications most commonly observed in children and young adults include speech and cognitive disabilities, developmental delays, abnormal motor function, and premature ovarian insufficiency in females (1). Despite its restriction from the diet, galactose remains present in patients with CG due to endogenous production, which occurs at significantly higher rates in children than adults (1, 4). A global comparison of CG treatments representing eleven countries found that there was no discernable relationship between differences in treatment and long-term outcomes; negative outcomes occurred in the majority of patients regardless of time of treatment initiation, rigor of galactose restriction, and level of

patient monitoring (5). A later study confirmed that there is no correlation between rigor of non-dairy galactose restriction and long-term outcome severity among patients (6).

Prenatal diagnosis of galactosemia has been possible since the 1970s, with the methods for detection including GALT assays in cultured amniotic cells, galactitol estimation in amniotic fluid supernatant, and estimation of UDP-galactose levels in amniotic cells (7, 8). Despite the ability to diagnose galactosemia prenatally by measuring galactose metabolite levels and enzyme activity, little is known about the metabolic profile of GALT-null fetuses through gestation. This is an important gap in knowledge with implications for potential prenatal origins of the negative outcomes that only present clinically as the child grows.

Previous studies have suggested that prenatal exposure to galactose may cause the premature ovarian insufficiency observed in galactosemia, and that the cause of this premature ovarian insufficiency phenotype may be the result of high galactose levels impairing germ cell migration (9, 10). A systematic review of existing literature concluded that prenatal galactose exposure is toxic to primordial and primary ovarian follicles and that postnatal galactose exposure is toxic to pre-antral and antral follicles, which may explain the premature ovarian insufficiency phenotype of galactosemia (11). Another study demonstrated that Gal-1P and galactitol levels of galactosemic fetuses were elevated in cord blood red blood cells and amniotic fluid even with a galactose-free maternal diet. This study also proposed that prenatal deficiency of myo-inositol resulting from the high levels of Gal-1P and galactitol in prenatal tissues may be responsible for the central nervous system (CNS) impairments associated with galactosemia (12). Another study demonstrated that galactose feeding resulted in elevated

galactitol levels, decreased aldose reductase mRNA levels, and decreased inositol levels in adult rat renal papillae (13).

The Leloir Pathway of Galactose Metabolism

The GALT enzyme, which is absent, non-functional, or minimally functional in patients with CG, plays an important role in the Leloir Pathway of galactose metabolism (Figure 1). The Leloir Pathway describes the process responsible for converting galactose to glucose, an inversion of just one carbon which makes the carbohydrate a more accessible source of energy for the body (14). Since galactose is produced endogenously as well as obtained from an individual's diet, this pathway's mechanisms are important to understand even for individuals with CG who follow a galactose-restricted diet (4). The primary enzymes in this pathway are galactose mutarotase, galactokinase (GALK), GALT, and UDP-galactose 4' epimerase (GALE) (14). CG is defined by the inheritance of two profoundly pathogenic alleles of *GALT*, however mutations to the *GALK* and *GALE* genes also occur and result in variant forms of galactosemia (15). As illustrated in Figure 1, galactose mutarotase catalyzes the epimerization of β-D-galactose to α-D-galactose, then GALK phosphorylates α-D-galactose to yield Gal-1P. GALT catalyzes the formation of glucose-1-phosphate (Glc-1P) and UDP-galactose (UDP-gal) from Gal-1P and UDP-glucose (UDP-glu), then finally GALE converts UDP-gal back to UDP-glu (14).

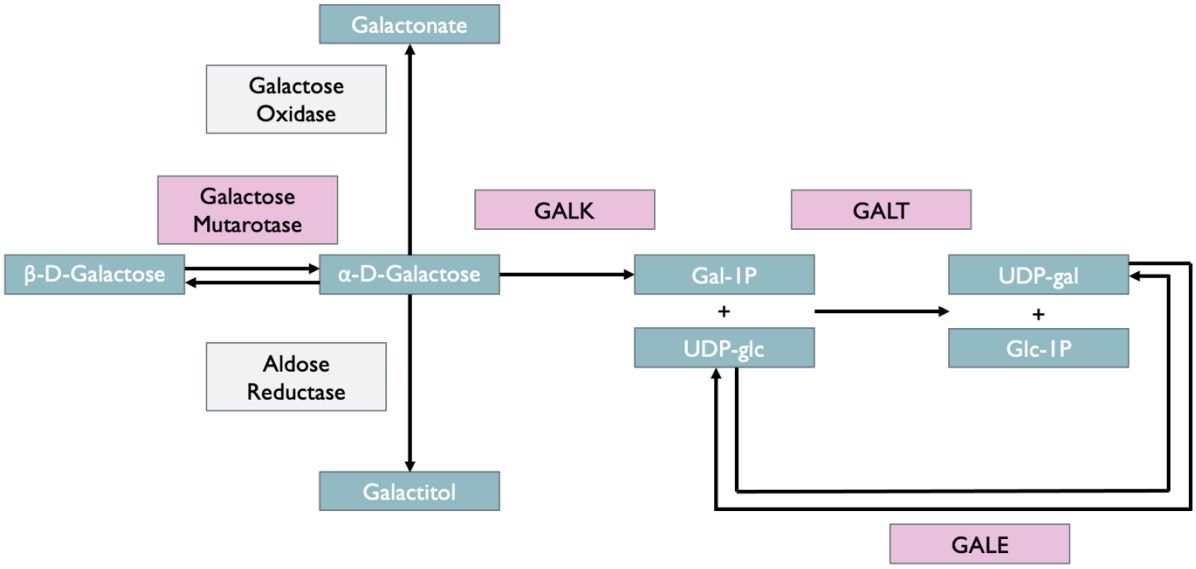


FIGURE 1. The Leloir Pathway of galactose metabolism. Metabolites of the pathway are represented with blue boxes, enzymes of the pathway are represented with pink boxes, and enzymes which interact with the metabolites but are not part of the pathway are represented with grey boxes.

An alternative to the Leloir Pathway for galactose metabolism is the conversion of galactose to galactitol by aldose reductase, resulting in abnormally elevated levels of galactitol in patients with CG (16). In some tissues, galactose can also be oxidized to form galactonate (4). The end product of the Leloir Pathway, UDP-glu, is important for enzymatic carbohydrate biosynthesis as a glycosyl donor as well as glycogen synthesis as a substrate (17).

Rat Estrous Cycles and Gestation

The female rat's reproductive cycle consists of four phases, each of which last approximately one day. These phases are proestrus, estrus, metestrus, and diestrus (18). An adult female rat's stage of estrus can be determined by performing a vaginal smear then using a

crystal violet stain to visualize the cell types under a microscope. The three cell types present are leukocytes, nucleated epithelial cells, and cornified epithelial cells (Figure 2) (19).

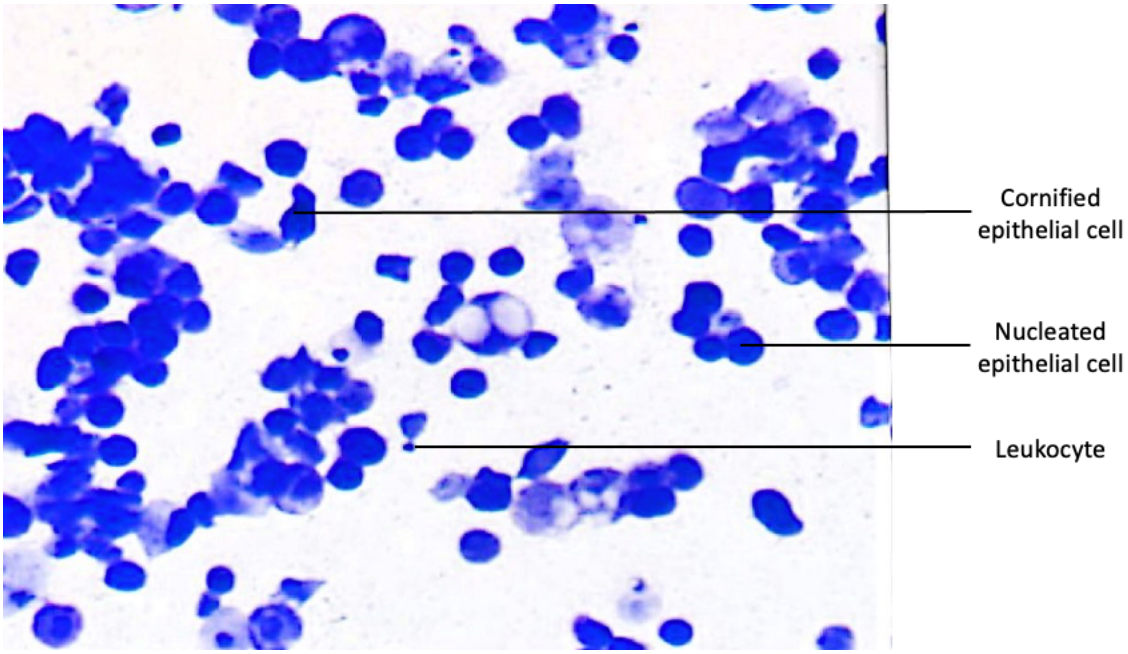


FIGURE 2. Depiction of the three cell types in vaginal smears. This image depicts a vaginal smear of a Sprague Dawley rat on proestrus.
The slide depicted in this image was prepared by Lauren Anshen of the Fridovich-Keil Lab.

Proestrus is characterized by primarily nucleated epithelial cells with a small proportion of leukocytes and cornified epithelial cells, and a relatively low overall cell count. Estrus is characterized by primarily cornified epithelial cells with a small proportion of nucleated epithelial cells, and a high overall cell count. Metestrus is characterized by the presence of all three cell types, with a particularly high proportion of leukocytes that tend to clump together. Diestrus is characterized by a large proportion of non-clumping leukocytes with smaller numbers of nucleated epithelial cells (19). Ovulation occurs at night between the proestrus and estrus stages, making females most receptive to males and thus most likely to get pregnant

when paired during the proestrus stage (20). Female rats reach puberty about fifty days after birth and the average gestational period of a female rat is 21-23 days (19, 21).

The CG Rat Model

The Fridovich-Keil Lab of Emory University collaborated with the Geurts Lab at the Medical College of Wisconsin to create a GALT-null Sprague Dawley rat as a model for CG using CRISPR-Cas9 gene editing. The mutation created, M3, resulted from non-homologous end joining at a double-stranded cut targeted to the gene locus encoding the active site of the rat GALT enzyme. After the successful introduction of this mutation into a male rat was confirmed by Sanger sequencing, the rat was outbred to create the SD-GALT-M3 strain which is still used by the Fridovich-Keil lab. This frame-shift GALT-null mutation is referred to as the M3 allele. Enzyme activity testing confirmed that GALT activity was undetectable in M3/M3 rat livers and about half the wild-type (WT) level in M3/WT livers, while GALK and GALE activities remained unaffected. These GALT-null rats demonstrated some phenotypes similar to human patients with CG including pre-pubertal growth delays, cataract formation, and subtle deficits in motor and cognitive functions. Accumulation of galactose, galactitol, and Gal-1P to varying degrees in different tissues and at all post-natal ages tested was also observed (22).

Excess Neonatal Demise in M3/M3 by M3/M3 Crosses

After the SD-GALT-M3 strain was successfully created and confirmed, one of the characteristics that the Fridovich-Keil Lab monitored was the success rates of crosses by genotype. While there was no significant difference in the success rates of crosses among young WT/WT, M3/WT, and M3/M3 mothers in terms of litter size at birth, there were

significant differences in neonatal demise among GALT-null pups based on parental genotypes. Nearly 90% of GALT-null pups from 30 M3/M3 by M3/WT crosses, regardless of whether the M3/M3 genotype was maternal or paternal, survived to weaning. In contrast, only about 40% of GALT-null pups from 34 M3/M3 by M3/M3 crosses survived to weaning (22).

From these observations, we hypothesize that fetal genotype alone may not control galactose metabolism, and that maternal genotype and/or the genotypes of other fetuses co-gestating in the same uterus may impact the severity of metabolic abnormalities in a GALT-null fetal rat, with consequences for post-natal survival. This project aims to determine if prenatal differences in metabolite levels correlate with the contexts where we have observed excess neonatal demise namely in GALT-null pups from M3/M3 by M3/M3 crosses.

In order to address this question, we performed tissue collections at four gestational timepoints from a variety of cross combinations, then extracted and quantified metabolites from the fetal livers, brains, and amniotic fluids to analyze their levels of galactose, galactitol, and Gal-1P. This allows for the development of a general overview of prenatal metabolites at different points in gestation between WT, heterozygous, and GALT-null fetuses as well as addresses the question of whether there may be some sort of cross-correction that reduces metabolite accumulation in GALT-null fetuses gestating among heterozygous siblings or within a heterozygous mom. The following table describes the crosses generated and their outcomes for each gestational timepoint (Table 1).

TABLE 1. Demographic Information of Entire Sample Population.

Litter FKRC	GD	Maternal Genotype	Paternal Genotype	WT/WT Fetuses	M3/WT Fetuses	M3/M3 Fetuses
331	15	M3/WT	M3/M3	0	8	4
357	15	WT/WT	WT/WT	11	0	0
401	15	M3/M3	M3/WT	0	7	7
410	15	M3/M3	M3/M3	0	0	15
267	17	M3/WT	M3/M3	0	6	3
269	17	M3/WT	M3/M3	0	2	6
344	17	WT/WT	WT/WT	12	0	0
398	17	M3/M3	M3/M3	0	0	5
402	17	M3/M3	M3/WT	0	9	5
405	17	M3/M3	M3/M3	0	0	15
268	19	M3/WT	M3/M3	0	1	5
270	19	M3/WT	M3/M3	0	5	5
330	19	WT/WT	WT/WT	12	0	0
343	19	M3/M3	M3/M3	0	0	10
366	21	WT/WT	WT/WT	13	0	0
377	21	M3/M3	M3/WT	0	5	10
378	21	M3/M3	M3/WT	0	9	6
385	21	M3/WT	M3/M3	0	11	2
386	21	M3/WT	M3/M3	0	8	6
397	21	M3/M3	M3/M3	0	0	14
399	21	M3/M3	M3/M3	0	0	15

To be clear, we have generated a great many samples that will yield answers to multiple questions. However, due to limitations with access to the high-performance liquid chromatography instrument (HPLC) needed to run these samples, we do not yet have results from all the samples described. We prioritized a subset of samples, and these will be the ones I describe in the chapters below. The remaining samples will be analyzed in the coming months and the data combined to yield a manuscript for publication.

CHAPTER 2: CHANGES IN METABOLITE LEVELS THOUGH GESTATION IN GALT-NULL RAT FETUSES WITH HETEROZYGOUS MOTHERS

Introduction

Previous studies have suggested that accumulation of metabolites from the Leloir Pathway may be responsible for at least some of the adverse phenotypes associated with CG (9, 10, 11, 12). The aim of this investigation was to characterize the metabolite levels of GALT-null fetuses through gestation to better understand the degree of abnormal metabolite accumulation in fetal tissues. To address this goal, tissue collections were performed on pregnant rats on gestational days (GDs) 15, 17, 19, and 21 to harvest fetal livers, brains, and samples of amniotic fluid. The pregnant rats included in this focused study were M3/WT females crossed with M3/M3 males. Litters were therefore anticipated to include about half M3/M3 and half M3/WT fetuses. Metabolite extractions were performed on each of the three harvested fetal tissues, then the samples were run through the HPLC to quantify the amount of each metabolite present in each tissue. Since CG is a rare autosomal recessive disease and premature ovarian insufficiency is very commonly observed in females with CG, most human babies born with the disease have carrier mothers (1). To parallel that experience, this section focuses on the changes in metabolite levels of M3/M3 fetuses from M3/WT (maternal) by M3/M3 (paternal) crosses using M3/WT fetuses from the same crosses as controls for comparison. To be clear, among human families affected by CG, typically both parents are carriers. Here, we used M3/M3 homozygous fathers to increase the yield of fetuses with the needed genotypes: GALT-null M3/M3 fetuses and GALT+ M3/WT fetuses. Of note, CG is an

autosomal recessive disorder and no significant metabolic differences are anticipated, or have been seen between M3/WT and WT/WT rats (22).

Methods

All methods involving live rats were performed using procedures approved by the Emory Institutional Animal Care and Use Committee (IACUC) under protocol 201700095. All live rats were fed LabDiet 5053, which derives 1% of its calories from galactose.

Rat Estrus Cycling and Crosses

Rat estrus cycling was performed using a modified version of the stained vaginal smear protocol detailed in Cora et al (23). 200 µL of 0.9% saline solution was drawn into a pipette then the pipette was gently placed 2-5 mm into the vaginal orifice of the female rat and the saline was flushed into and out of the vagina 2-3 times. The saline solution was then dispensed into a 1.5 mL microfuge tube. The tube was held to the light and flicked to determine if there was an adequate amount of cloudiness to continue. If there was no visible cloudiness to the sample, then the procedure was repeated with a new aliquot of saline solution (23). Care was taken to avoid inserting the pipette tip too deep into the vaginal orifice to avoid injuring the rat and because this can cause pseudopregnancy characterized by persistent diestrus and a physically pregnant appearance (24).

The tubes containing the saline cell suspensions were centrifuged for approximately five seconds in order to form a pellet of cells at the bottom of the tube. About 150 µL of the saline solution, or whatever volume caused about 20-30 µL to remain in the tube depending on how

much solution was lost during the vaginal collection, was removed and discarded. The remaining solution was pipetted up and down 2-3 times to resuspend the cell pellet, then about 10 µL of the suspension were pipetted onto a glass slide and spread into a dime-sized circle. These slides were allowed to air-dry near a fan for at least ten minutes. Once dry, 0.1% Crystal Violet stain was applied to the slides for one minute then the slides were rinsed with running water and allowed to airdry for 5-10 minutes. The slides were visualized under a 10x objective lens on a microscope and the current stage of the estrous cycle was determined according to the total cell counts and types of cells present as described in Cora et al (23). Slides were then sealed with Thermo Scientific Cytoseal, stored, and arranged by date for future reference.

All vaginal lavage samples were collected between 10 am and 12 pm, and the slides were stained and interpreted immediately thereafter. Mating pairs were set up when the female was on proestrus or transitioning from proestrus to estrus by 2 pm of the day that sample was collected. Females remained paired with males for 24 hours then estrus cycling was performed again in the same manner as described above, but the slide was also studied for the presence of sperm. If the female was sperm negative, the estrus cycling procedures were continued. If the female was sperm positive, then she was separated from the male and the day her sample was sperm positive was counted as GD 1. All paired females, including those whose samples appeared sperm negative, were monitored for weight gain to determine if a pregnancy was progressing.

Gestational Tissue Collections

Timed gestational tissue collections were performed on GDs 15, 17, 19, and 21 with the day that female rat tested sperm positive being considered GD 1. The pregnant rats were deeply anesthetized by isoflurane inhalation and sprayed with 70% ethanol prior to performing a U-shaped incision in the chest cavity to expose the heart. Blood was collected from the right atrium with a 25-gauge syringe then deposited into a sodium heparin BD Vacutainer tube (#366480), inverted 8-10 times, then stored on ice until processing (22). After the heart was cut, the rat was exsanguinated. Blood was subsequently processed into plasma and red blood cell (RBC) components as described below. The other maternal tissues collected were liver, brain, ovaries, eyes, and uterine horn. These tissues were placed in tubes that were flash frozen on dry ice for later analyses.

After waiting for at least five minutes, the fetuses were euthanized by loss of blood flow from the dam and maternal exposure to isoflurane. The uterine horns were removed from the dam and fetuses were numbered starting on the right such the fetus closest to the right ovary was number one and the fetus closest to the left ovary was the last number (Figure 3).

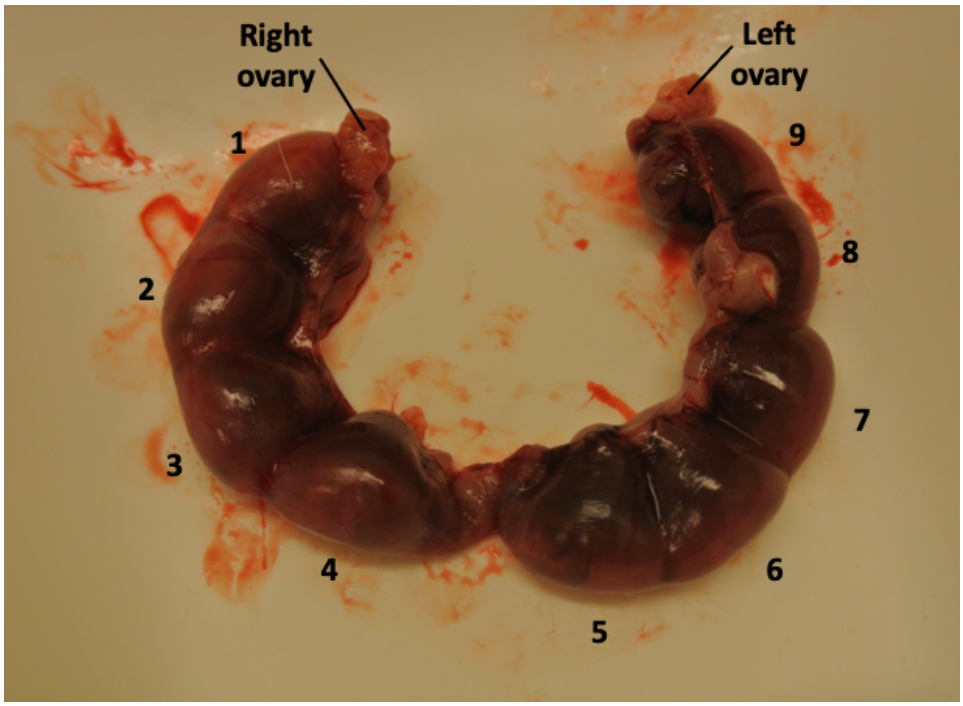


FIGURE 3. Intrauterine positioning of fetuses. Fetuses pictured were harvested on GD 17. This numbering system was used to ensure that the tissues of each animal could be specifically identified, which was particularly important for litters anticipated to include a mix of M3/WT and M3/M3 fetuses.

After the uterine horns were removed from the dam, each fetus was separated and placed in pre-labeled weigh boats on ice. A 25-gauge syringe was used to draw up amniotic fluid, which was then pipetted into a tube and placed on ice. After the amniotic sacs were removed, the placentas were separated from the fetuses and placed in tubes (Figure 4).

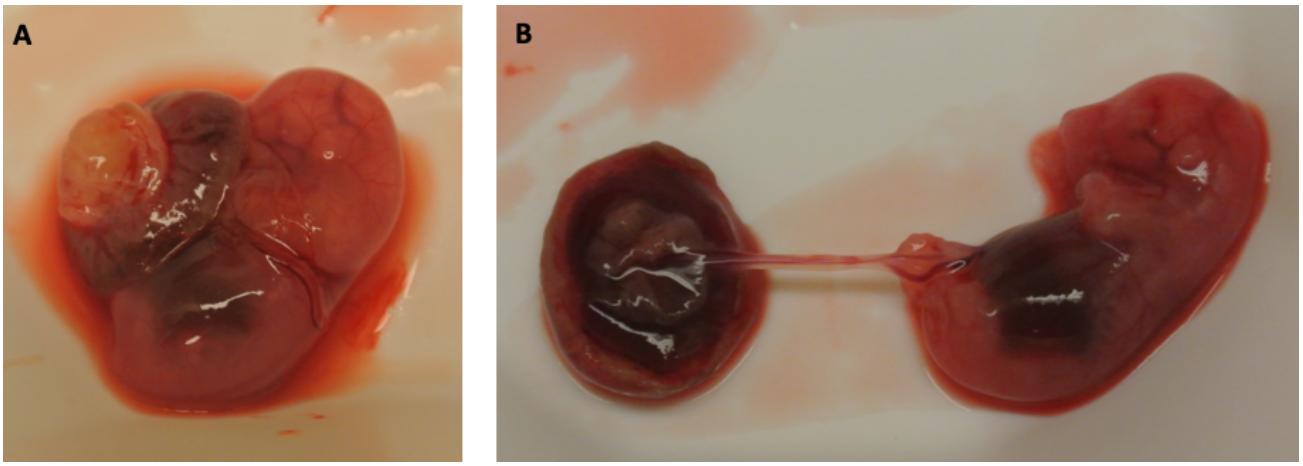


FIGURE 4. Images of fetuses after harvesting. A depicts a fetus still in the amniotic sac after amniotic fluid was drained. B depicts a fetus after removal of the amniotic sac. Fetuses pictured were harvested on GD 19.

The whole body and placentas were weighed. The fetal livers, brains, and tails were also collected then all these tubes were flash frozen on dry ice.

The blood was transferred from the vacutainer tube into two 1.5 microfuge tubes, then both the blood and amniotic fluid tubes were centrifuged in a 4 °C room at 2000 revolutions per minute (RPM) for 15 minutes. For the amniotic fluids, this causes a pellet composed of cell debris to form at the bottom of the tube. The remaining fluid was aliquoted into new tubes, one containing a 100 µL aliquot and the other containing the remaining amount, while the pellet remained in the original tube. For the blood, centrifugation separates the plasma from the RBCs. The top plasma layer was removed from the top of the tube and aliquoted into a new tube, then approximately 1000 µL of 1X Phosphate Buffered Saline (PBS) (Corning 21-040-CV) was used to resuspend the RBC bottom layer. The RBC tube was again centrifuged in a 4 °C room at 2000 RPM for 15 minutes, then the PBS layer was discarded and the RBCs were aliquoted into new tubes (22). After the complete collection and processing of all tissues, all

samples excluding the tails were stored in a -80 °C freezer until metabolite extractions were performed. The tails were stored in the -20 °C freezer until they were sent to Transnetyx for genotyping.

Metabolite Extractions

Metabolite extractions were performed as described previously (22), modified slightly to accommodate the smaller size of prenatal rats and the addition of amniotic fluid as a tissue. For livers and brains, fifty to sixty milligrams of each tissue, or the entirety of the tissue if less than fifty milligrams, was aliquoted into a 1.5 mL microfuge tube. For amniotic fluid, 100 µL, or 50 µL if 100 µL were not obtained from the fetus, of amniotic fluid was aliquoted into a 1.5 mL microfuge tube. All remaining portions of the metabolite extraction protocol were identical for livers, brains, and amniotic fluids, which will now be referred to only as tissue. 125 µL of ice-cold HPLC-grade water was added to each tube with the tissue, then the tissues were ground with a small, motorized pestle for twenty seconds each. 250 µL of HPLC-grade 100% methanol (MeOH) and 500 µL of HPLC-grade chloroform ($CHCl_3$), both chilled to -20 °C, were added to each tube. Then, the samples were agitated on a vortex mixer for 45 minutes and centrifuged at 2000 RPM for 20 minutes at 4 °C. The top aqueous layer was removed into a new 1.5 mL microfuge tube and placed on ice. 250 µL of a 1:1 mixture of HPLC-grade water and HPLC-grade 100% MeOH chilled to -20 °C were added to each remaining bottom $CHCl_3$ layer. Re-extraction of the bottom layer was performed by vortex agitation of these tubes for 5 minutes then centrifuging at 2000 RPM for 20 minutes at 4 °C. After the re-extraction, the aqueous top layer from the original tube was removed and combined with the top layer in the second tube on the

ice. The remaining tube containing the bottom $CHCl_3$ layer was disposed of in the designated $CHCl_3$ chemical waste container. The top aqueous layer tube was dried in the SpeedVac with no heat, for about 2-4 hours or until no more fluid was present. Dried metabolite pellets were resuspended in 200 µL of ice-cold HPLC-grade water. The resuspension was filtered by being added to a Costar 8169 Spin-X centrifuge tube with a 0.22 um pore size, then being centrifuged at 4000 relative centrifugal force (RCF) at 4 °C. The filter was removed from the tube and samples were stored in a -80 °C freezer until ready for HPLC analysis (22).

Metabolite Quantifications

Metabolites were removed from the freezer and allowed to thaw on ice. Test dilutions of each tissue were prepared to determine which dilution was optimal for interpretation of HPLC results based on the standard curves. All metabolites were diluted with HPLC-grade water, as presented in the following tables.

TABLE 2. Dilutions used for amniotic fluid metabolites by genotype and GD of fetus.

Genotype	GD 15	GD 17	GD 19	GD 21
M3/M3	1	1	1	1:2
M3/WT	1	1	1	1:2
WT/WT	1	1	1	1

TABLE 3. Dilutions used for liver metabolites by genotype and GD of fetus.

Genotype	GD 15	GD 17	GD 19	GD 21
M3/M3	1:8	1:8	1:10, 1:5*	1:10, 1:5*
M3/WT	1:4	1:4	1:5	1:5
WT/WT	1:4	1:4	1:5	1:5

*1:10 dilution was used for fetuses with M3/M3 mothers, 1:5 dilution was used for fetuses with M3/WT mothers.

TABLE 4. Dilutions used for brain metabolites by genotype for GD 21 fetuses.

Genotype	GD 21
M3/M3	2:3, 1:2*
M3/WT	2:3
WT/WT	2:3

*1:2 dilution was used for fetuses from M3/M3 by M3/M3 crosses, 2:3 dilution was used for fetuses from M3/M3 by M3/WT crosses (both directions).

For tables 2-4, the dilution used is listed as parts metabolite: parts HPLC-grade water. A dilution of 1 indicates that the metabolite remained undiluted for HPLC analysis.

Galactose and galactitol were filtered through an amino trap column (4 x 50 mm) and borate trap column (4 x 50 mm) using 1 M NaOH (Buffer A) and 15 mM NaOH (Buffer B) on a CarboPac MA1 analytical column (4 x 250 mm). The CarboPac MA1 column used a flow rate of 0.4 mL/min with the following ratios of each buffer: 30% Buffer A and 70% Buffer B for -5 to 43 minutes, 70% Buffer A and 30% Buffer B for 43 to 45 minutes using linear increases and decreases from the starting ratio, then at hold at 70% Buffer A and 30% Buffer B for 45 to 50 minutes (22). The CarboPac PA10 analytical column (250 x 4 mm), with an amino-trap column

(50 x 14 mm) placed in front of the analysis column and a borate-trap column (50 x 4 mm) placed in front of injector port, was used to separate Gal-1P (22, 25). 15 mM NaOH (Buffer A) and 50 mM NaOH/1M NaAC (Buffer B) mobile phase buffers were used. A 50% sodium hydroxide solution was added while preparing the buffers to prevent carbonate contamination of the analysis column (25). The CarboPac PA10 column used a flow rate of 0.8 mL/min with the following ratios of each buffer: 90% Buffer A and 10% Buffer B for -5 to 1 minutes, linear increase of Buffer B to 65% for 1 to 30 minutes, hold of Buffer B at 65% for 30 to 52 minutes, then a linear decrease of Buffer B back to 10% for 52 to 54 minutes. The injection volume for all samples on both columns was 20 µL, and the autosampler tray was chilled in a -20 °C prior to loading of the samples.

The liver and brain metabolite samples were run on both the MA1 column and the PA10 column in order to quantify the galactose, galactitol, and Gal-1P levels for these tissues. The amniotic fluid samples were only run on the MA1 column because Gal-1P is an intracellular metabolite, so it should not be found in amniotic fluid, which is extracellular (26). A small group of M3/M3 amniotic fluid metabolites, which would be expected to contain the highest levels of Gal-1P if present, were run on the PA10 column to confirm that there was no Gal-1P present in any of these samples. Since this test group did indeed show no discernable Gal-1P levels, the remaining amniotic fluid samples were only run on the MA1 column to quantify the galactose and galactitol levels.

The PA10 standards run to create the standard curve for the PA10 column were 1x, 5x, and 10x dilutions. A 10x stock solution of the PA10 standard consists of 50 µL of 10 nmol/µL

galactose, 50 µL of 10 nmol/µL glucose, 400 µL of 10 nmol/µL Gal-1P, 100 µL of 10 nmol/µL Glc-1P, 100 µL of 10 nmol/µL fructose-1-phosphate (Fruc-1P), 50 µL of 10 nmol/µL fructose-6-phosphate (Fruc-6P), 100 µL of 10 nmol/µL glucose-6-phosphate (Glc-6P), 100 µL of 10 nmol/µL galactonate, 50 µL of 10 nmol/µL UDP-gal, 100 µL of 10 nmol/µL UDP-glc, 5 µL of 20 nmol/µL UDP N-acetylgalactosamine (UDPgalNac), 10 µL of 10 nmol/µL UDP N-acetylglucosamine (UDPglcNac), and 8885 µL of HPLC-grade water.

The MA1 standards run to create the standard curve for the MA1 column were 1x, 2.5x, 5x, and 10x dilutions. A 10x stock solution of the MA1 standard consists of 50 µL of 10 nmol/µL myo-inositol, 100 µL of 10 nmol/µL galactitol, 50 µL of 10 nmol/µL glucose, 50 µL of 10 nmol/µL galactose, and 9750 µL of HPLC-grade water. With each run, the HPLC generated a chromatogram depicting the elution times of each component of the sample. The peaks for the metabolites of interest were identified based on the elution time of the standards as well as the location of the peaks of interest relative to other identifiable peaks. For each metabolite, the peaks were integrated then the Chromeleon software automatically calculated the area under the peak. The Chromeleon software then calculated the pmol of metabolite in the sample based on the integrated peaks and the standard curve for that metabolite.

After the pmol of metabolite was calculated, this value needed to be standardized to compare between samples. For livers and brains, it was standardized to pmol of metabolite per mg tissue based on the following calculation: pmol of metabolite in sample * dilution factor * resuspension volume (200 µL) / injection volume (20 µL) / mg tissue. For amniotic fluids, it was standardized to pmol of metabolite per µL amniotic fluid based on the following calculation:

pmol of metabolite in sample * dilution factor * resuspension volume (200 µL) / injection volume (20 µL) / µL amniotic fluid.

Sample Size

Due to time constraints and the reality of sharing an HPLC between multiple projects, metabolite quantitation data has only been collected for a subset of the total samples. Due to prioritization of certain samples and the fact that amniotic fluid could be analyzed on the HPLC at the same time as samples from another project which run on the PA10 column only, while liver and brain could not, the number of samples analyzed for each tissue is different. The following tables describe the samples analyzed for each tissue so far.

TABLE 5. Demographics of amniotic fluid samples analyzed in Chapter 2.

FKRC	n	GD	Fetus Genotype	Maternal Genotype	Paternal Genotype
331	8	15	M3/WT	M3/WT	M3/M3
331	4	15	M3/M3	M3/WT	M3/M3
267	3	17	M3/WT	M3/WT	M3/M3
269	2	17	M3/WT	M3/WT	M3/M3
267	6	17	M3/M3	M3/WT	M3/M3
269	6	17	M3/M3	M3/WT	M3/M3
268	1	19	M3/WT	M3/WT	M3/M3
270	5	19	M3/WT	M3/WT	M3/M3
268	5	19	M3/M3	M3/WT	M3/M3
270	5	19	M3/M3	M3/WT	M3/M3
385	9	21	M3/WT	M3/WT	M3/M3
386	6	21	M3/WT	M3/WT	M3/M3
385	1	21	M3/M3	M3/WT	M3/M3
386	4	21	M3/M3	M3/WT	M3/M3

TABLE 6. Demographics of liver samples analyzed in Chapter 2.

FKRC	n	GD	Fetus Genotype	Maternal Genotype	Paternal Genotype
331	4	15	M3/WT	M3/WT	M3/M3
331	4	15	M3/M3	M3/WT	M3/M3
267	3	17	M3/WT	M3/WT	M3/M3
269	1	17	M3/WT	M3/WT	M3/M3
267	4	17	M3/M3	M3/WT	M3/M3
268	1	19	M3/WT	M3/WT	M3/M3
270	3	19	M3/WT	M3/WT	M3/M3
268	4	19	M3/M3	M3/WT	M3/M3
385	4	21	M3/WT	M3/WT	M3/M3
385	2	21	M3/M3	M3/WT	M3/M3
386	4	21	M3/M3	M3/WT	M3/M3

TABLE 7. Demographics of brain samples analyzed in Chapter 2.

FKRC	n	GD	Fetus Genotype	Maternal Genotype	Paternal Genotype
385	4	21	M3/WT	M3/WT	M3/M3
385	2	21	M3/M3	M3/WT	M3/M3
386	4	21	M3/M3	M3/WT	M3/M3

Statistical Methods

In order to address the goal of gaining an overview of the metabolites present in fetuses of different genotypes through gestation, comparisons were made between M3/M3 and M3/WT fetuses from M3/WT (maternal) by M3/M3 (paternal) crosses, at every gestational timepoint and between the two fetal genotypes through gestation. All graphing and statistical calculations were performed in R Studio. The Kruskal-Wallis Test was used to determine overall significance at $\alpha = 0.05$ for graphs of metabolite levels though gestation. The Kruskal-Wallis Test was used rather than an analysis of variance (ANOVA) to account for the non-normal distribution of the data. Pairwise Wilcoxon Tests were then performed with the Benjamini and Hochberg correction adjustment method applied. The Benjamini and Hochberg correction was selected because it controls the false discovery rate (27). $\alpha = 0.05$ was also used to determine the significance of each pairwise comparison. For the graphs comparing M3/M3 metabolites to M3/WT metabolites, the Wilcoxon Test was performed and significance was determined at $\alpha = 0.05$.

Results

Metabolite Levels in M3/M3 and M3/WT Fetuses through Gestation: Amniotic Fluid

Galactitol levels in M3/M3 amniotic fluid showed a significant increase across all gestational timepoints except GD 17 to 19, while galactitol levels in M3/WT amniotic fluid showed a significant increase only from GD 19 to 21 only (Figures 5, 6, S1). Galactose levels in M3/M3 amniotic fluid showed no significant changes through gestation, while in M3/WT amniotic fluid galactose levels significantly decreased from gestational day 19 to 21 only

(Figures 7, 8, S2). Gal-1P levels were not measured in amniotic fluid because Gal-1P is an intracellular metabolite (26).

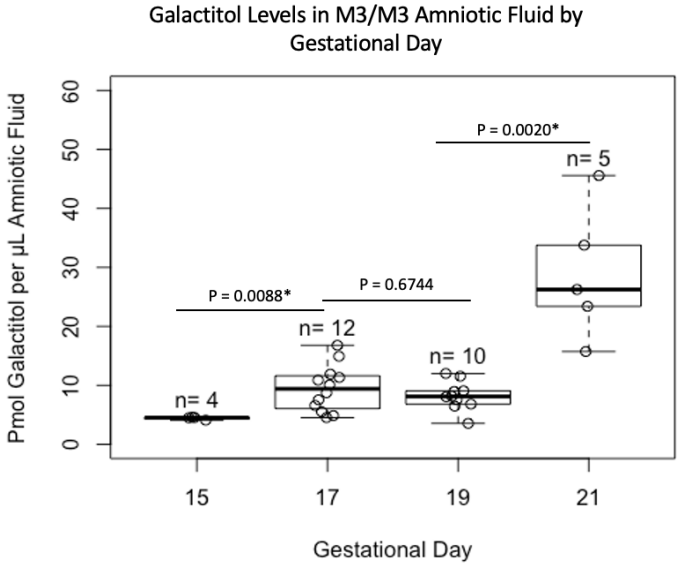


Figure 5. Galactitol Levels in M3/M3 Amniotic Fluid by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.0006468 *, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

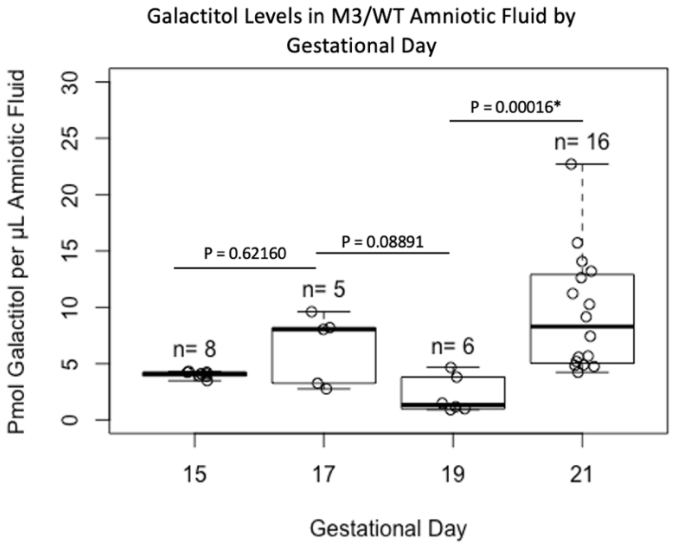


Figure 6. Galactitol Levels in M3/WT Amniotic Fluid by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.0001376*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

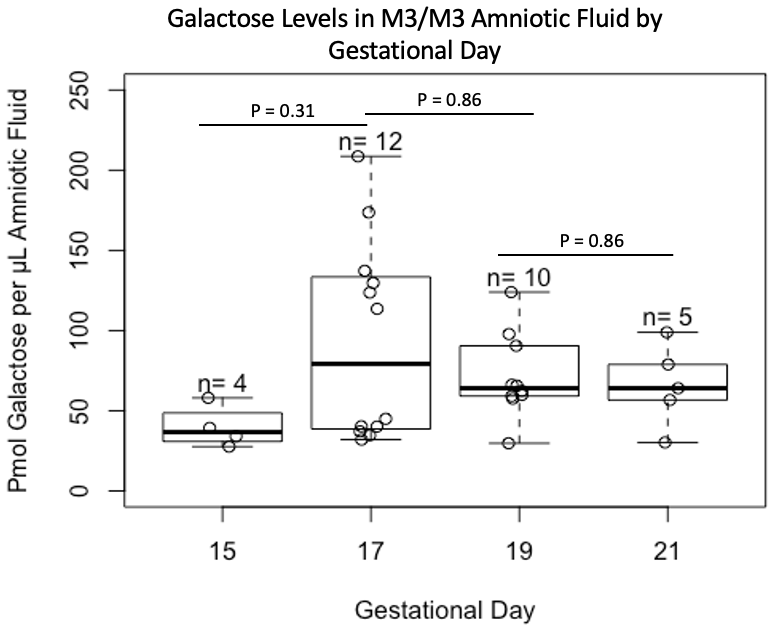


Figure 7. Galactose Levels in M3/M3 Amniotic Fluid by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.1913, pairwise comparison shown on graph.

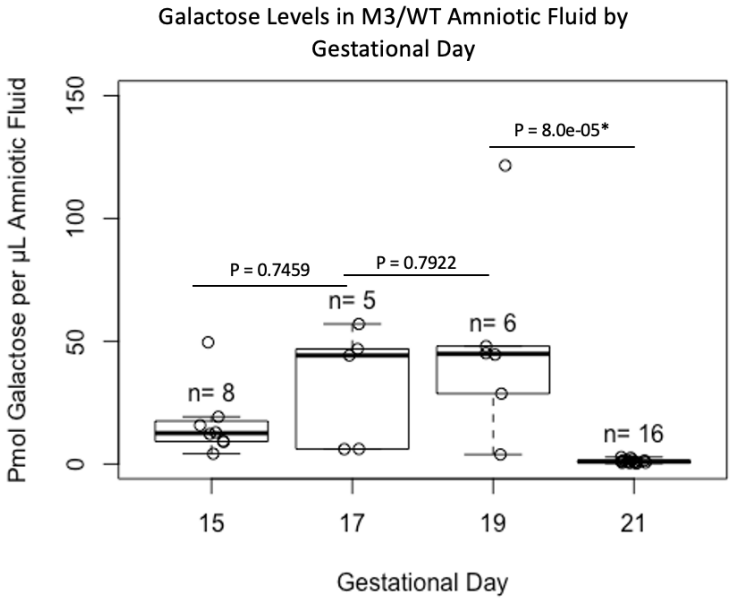


Figure 8. Galactose Levels in M3/WT Amniotic Fluid by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 9.738e-06*, pairwise comparisons shown on graph.

*Indicates significant p-value (P < 0.05).

Metabolite Levels in M3/M3 and M3/WT Fetuses through Gestation: Fetal Livers

Galactitol and galactose levels in M3/M3 livers showed significant increases through gestation (Figures 9, 11, S3, S4). Gal-1P levels in M3/M3 livers showed no significant differences between any consecutive gestational days (Figure 13, S5). Galactitol, galactose, and Gal-1P in M3/WT livers showed no significant changes through gestation (Figures 10, 12, 14, S3, S4, S5).

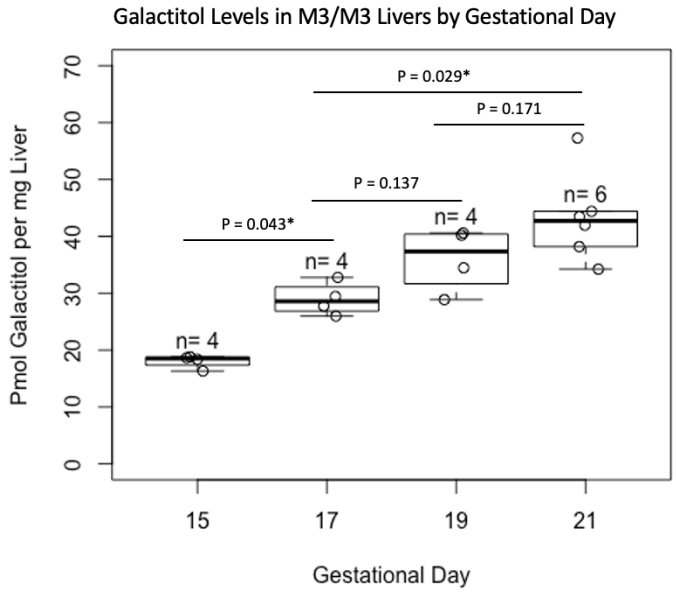


Figure 9. Galactitol Levels in M3/M3 Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.003184*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

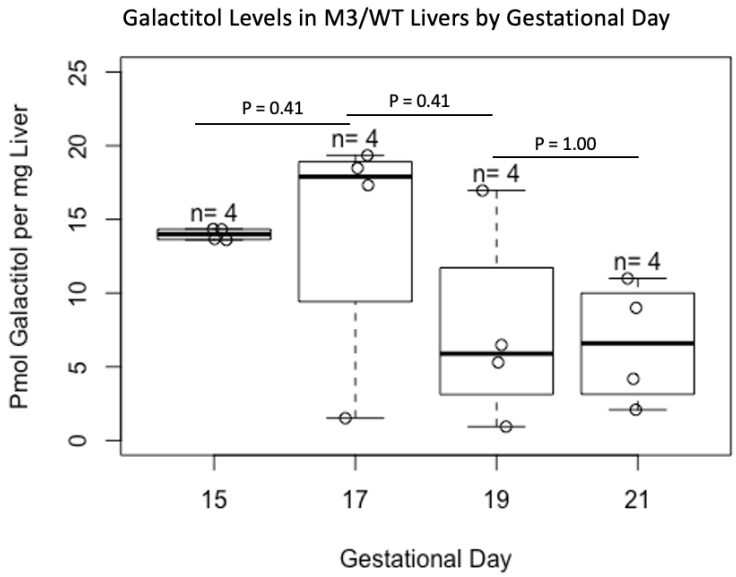


Figure 10. Galactitol Levels in M3/WT Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.1681, pairwise comparisons shown on graph.

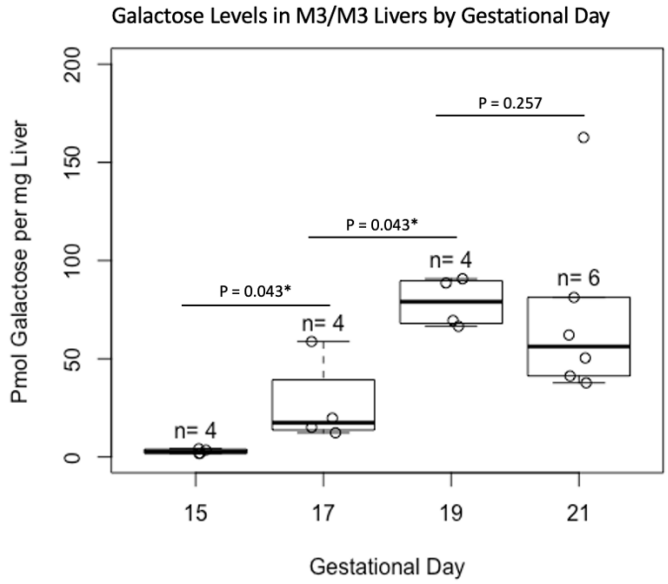


Figure 11. Galactose Levels in M3/M3 Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.004325*, pairwise comparisons shown on graph.

*Indicates significant p-value (P < 0.05).

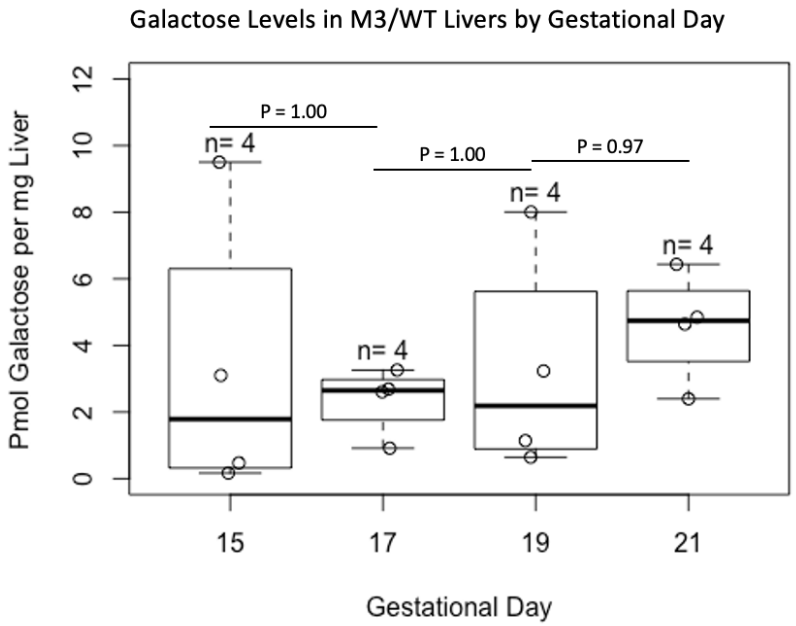


Figure 12. Galactose Levels in M3/WT Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.5894, pairwise comparisons shown on graph.

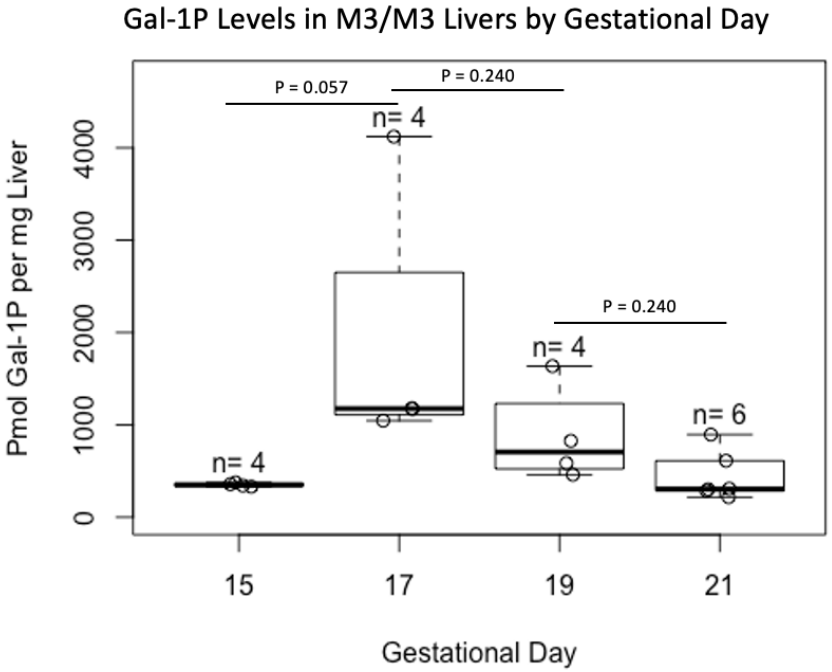


Figure 13. Gal-1P Levels in M3/M3 Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.01336*, pairwise comparisons shown on graph.

*Indicates significant p-value ($P < 0.05$).

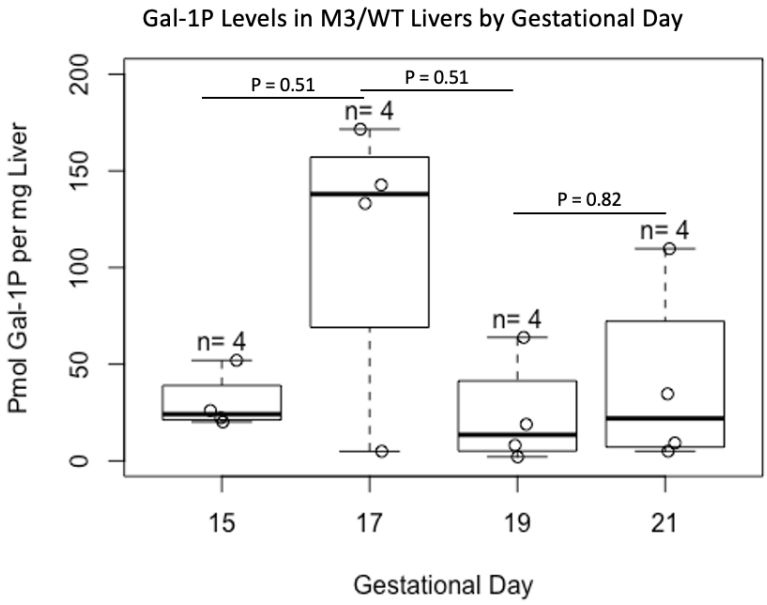


Figure 14. Gal-1P Levels in M3/WT Livers by Gestational Day. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses. Kruskal-Wallis Test P = 0.3464, pairwise comparisons shown on graph.

Fetal Metabolite Levels by GALT Genotype: GD 15

Galactitol and galactose levels in GD 15 amniotic fluid were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 15, 16). Galactitol and Gal-1P levels in GD 15 livers were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 17, 19). Galactose levels in GD 15 livers were not significantly different between M3/M3 and M3/WT fetuses (Figure 18).

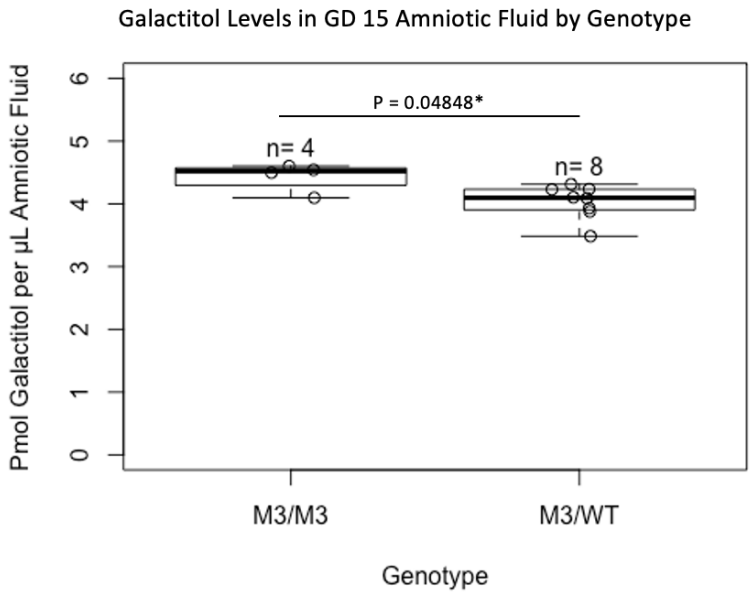


Figure 15. Galactitol Levels in GD15 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

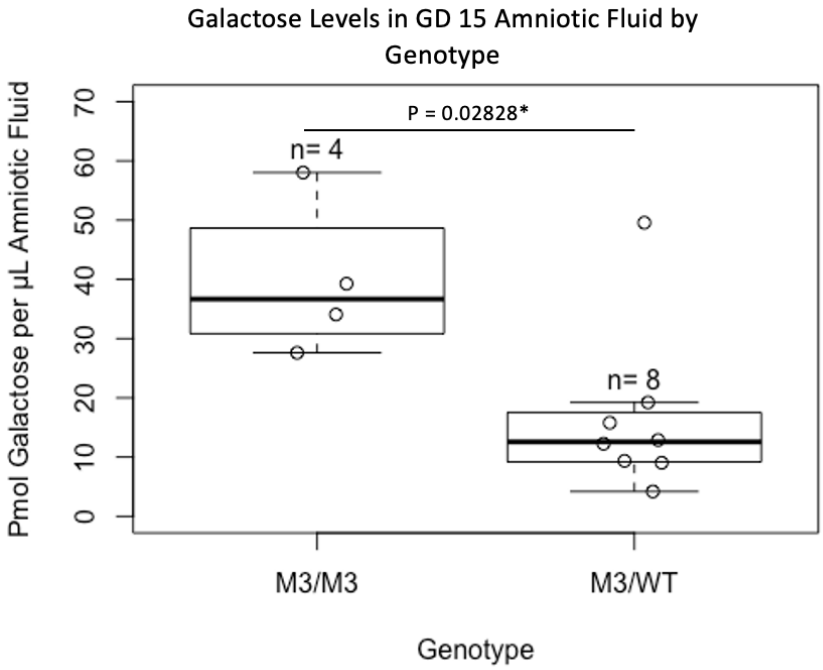


Figure 16. Galactose Levels in GD15 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

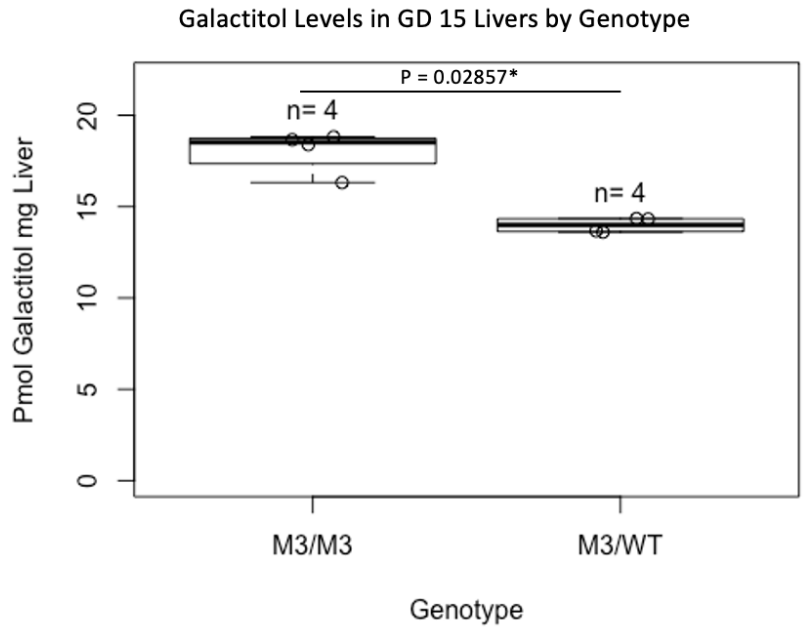


Figure 17. Galactitol Levels in GD15 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

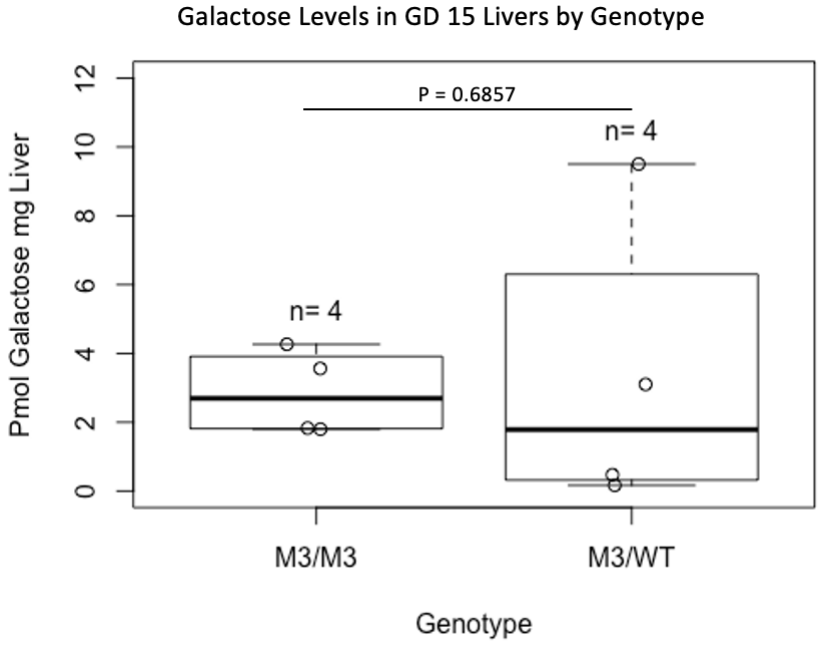


Figure 18. Galactose Levels in GD15 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.

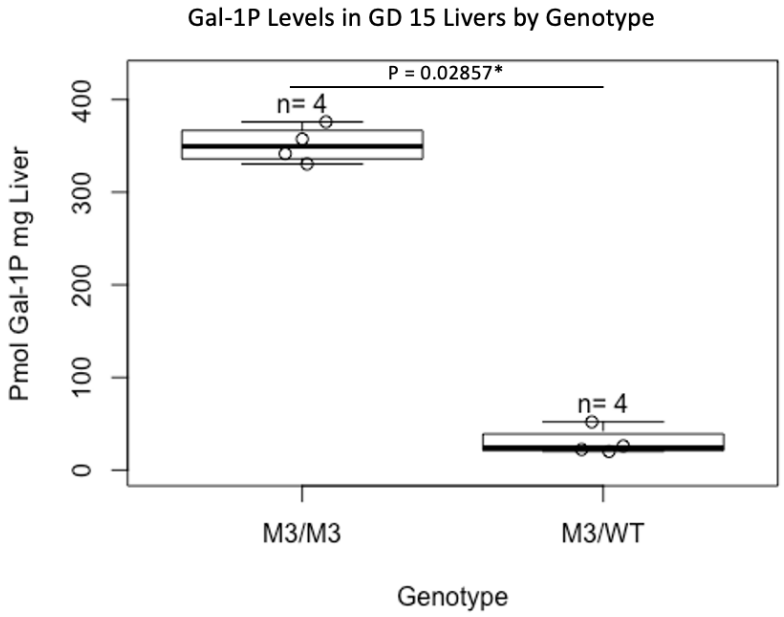


Figure 19. Gal-1P Levels in GD15 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value ($P < 0.05$).

Fetal Metabolite Levels by GALT Genotype: GD 17

Galactitol and galactose levels in GD 17 amniotic fluid were not significantly different between M3/M3 and M3/WT fetuses (Figures 20, 21). Galactitol, galactose, and Gal-1P levels in GD 17 livers were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 22, 23, 24).

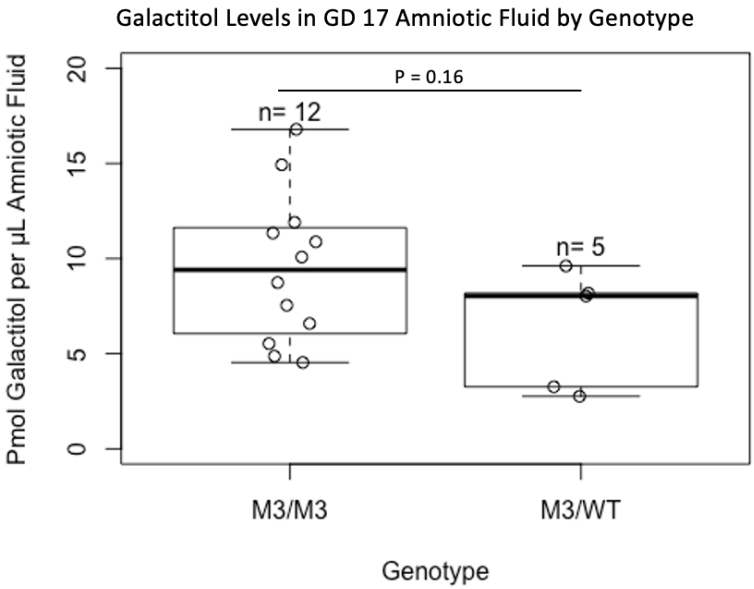


Figure 20. Galactitol Levels in GD17 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.

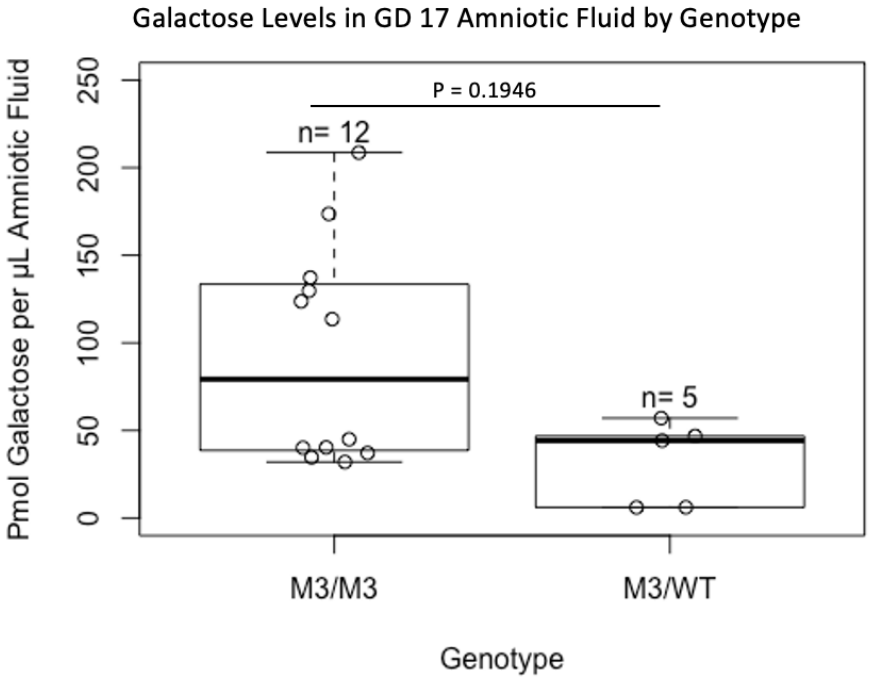


Figure 21. Galactose Levels in GD17 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.

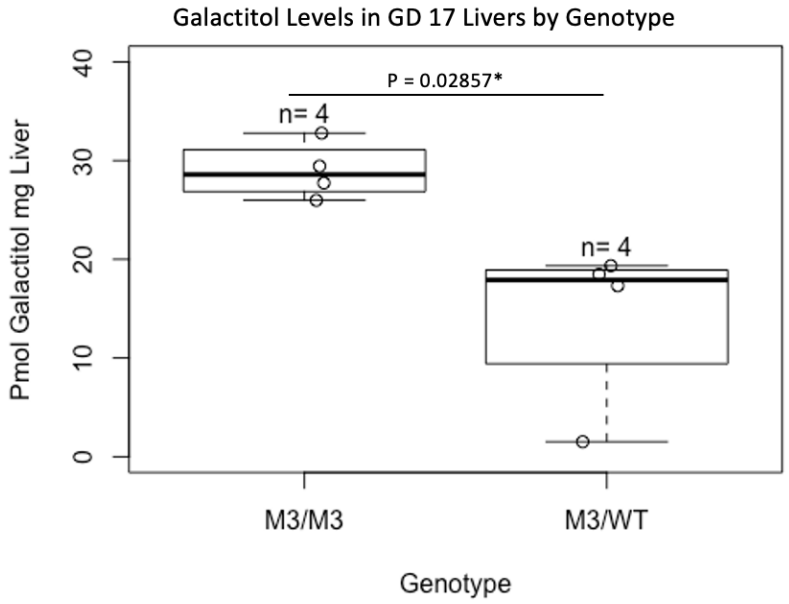


Figure 22. Galactitol Levels in GD17 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

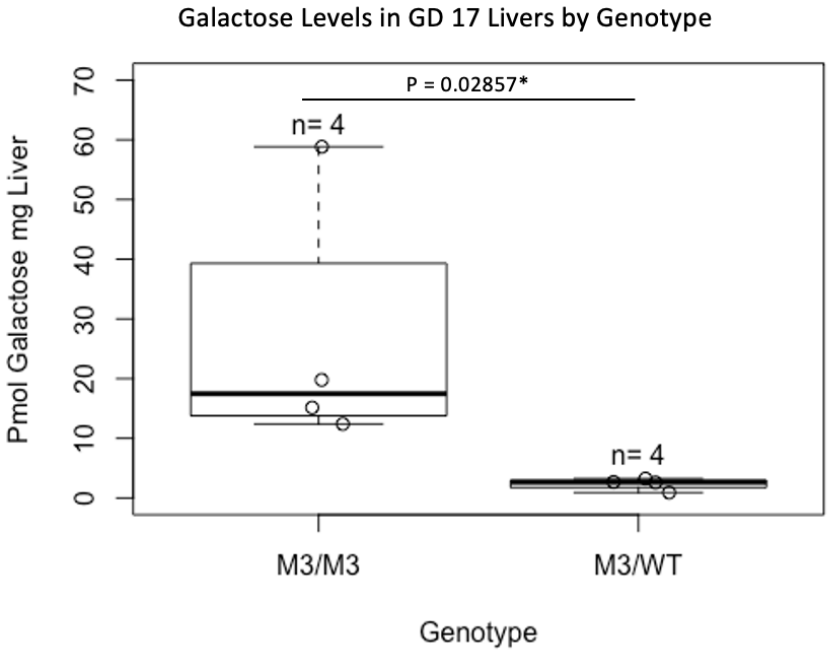


Figure 23. Galactose Levels in GD17 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

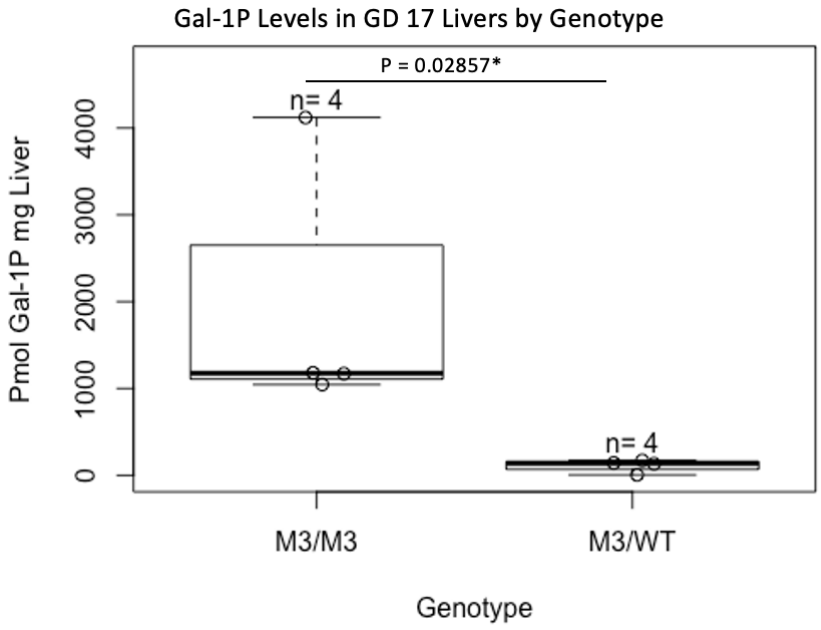


Figure 24. Gal-1P Levels in GD17 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value ($P < 0.05$).

Fetal Metabolite Levels by GALT Genotype: GD 19

Galactitol levels in GD 19 amniotic fluid were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figure 25). Galactose levels in GD 19 amniotic fluid showed no significant differences between M3/M3 and M3/WT fetuses (Figure 26). Galactitol, galactose, and Gal-1P levels in GD 19 livers were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 27, 28, 29).

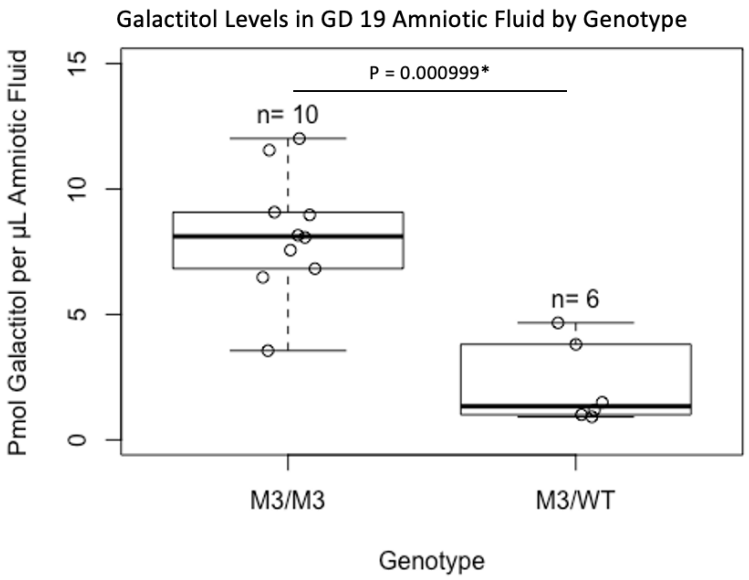


Figure 25. Galactitol Levels in GD19 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

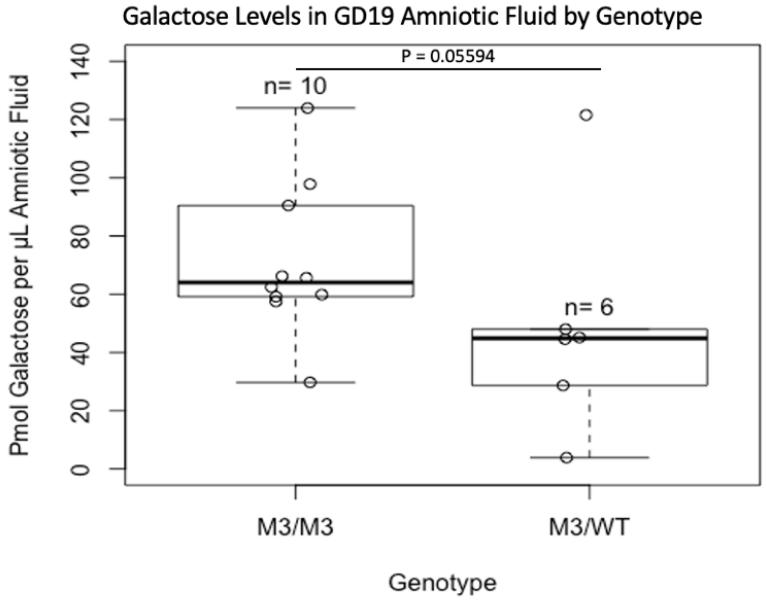


Figure 26. Galactose Levels in GD19 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.

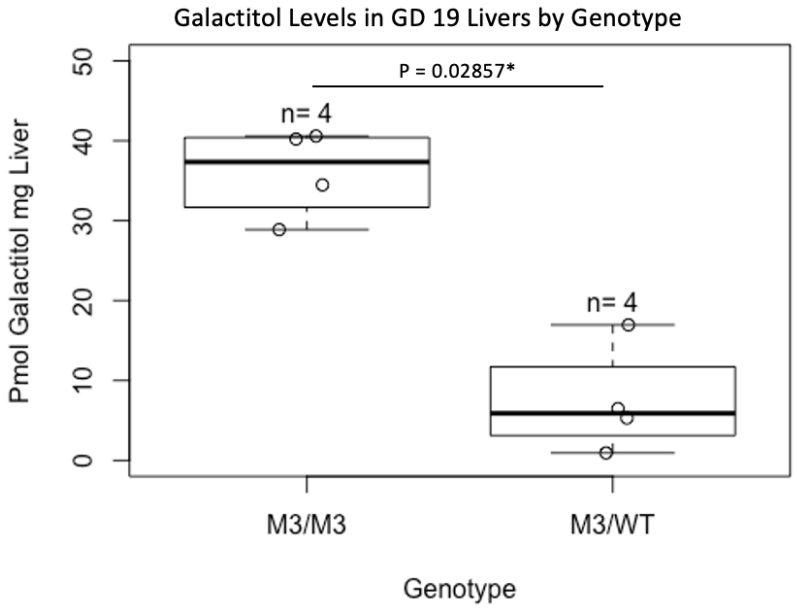


Figure 27. Galactitol Levels in GD19 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

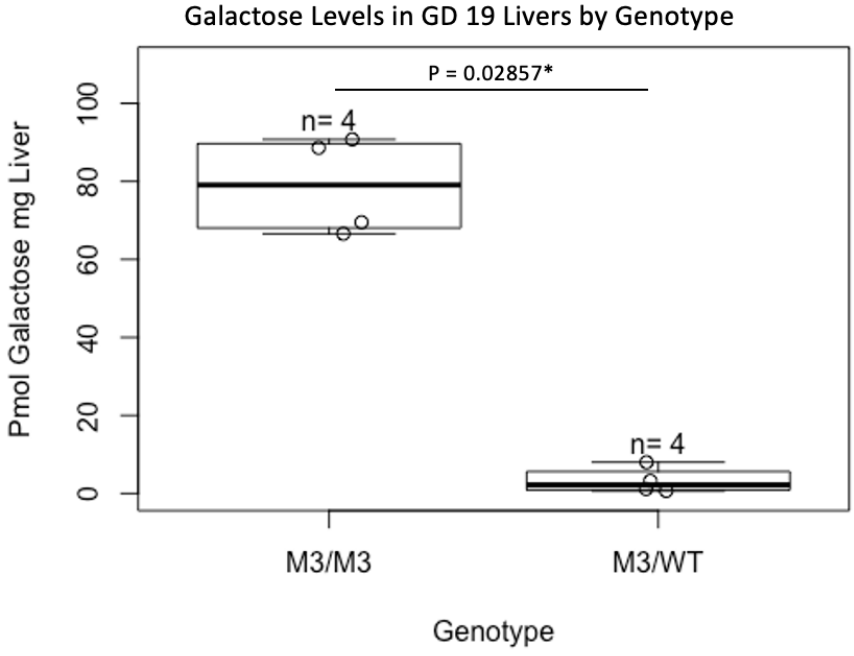


Figure 28. Galactose Levels in GD19 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

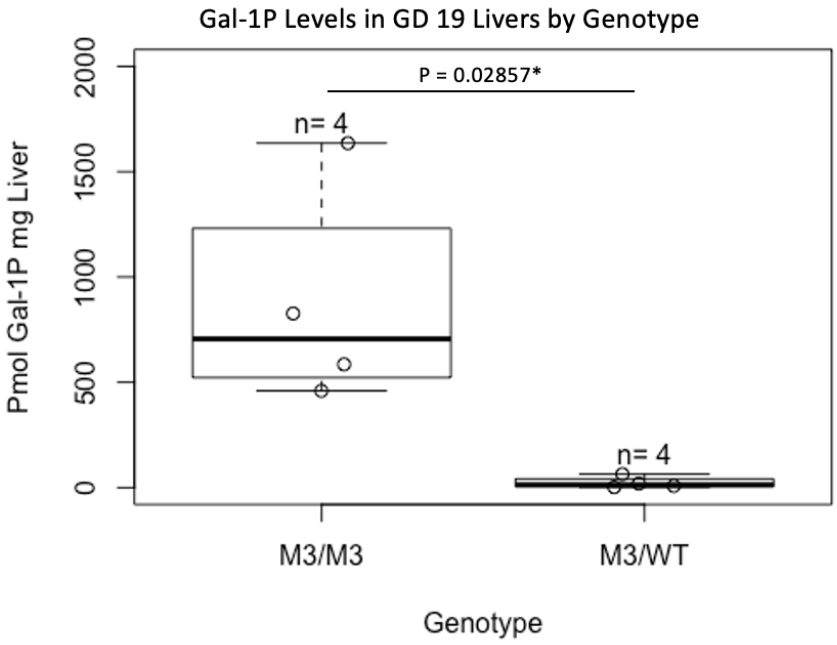


Figure 29. Gal-1P Levels in GD19 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value ($P < 0.05$).

Fetal Metabolite Levels by GALT Genotype: GD 21

Galactitol and galactose levels in GD 21 amniotic fluid were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 30, 31). Galactitol, galactose, and Gal-1P levels in GD 21 livers were also significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 32, 33, 34). Galactitol levels in GD 21 brains appeared slightly higher in M3/M3 fetuses than M3/WT fetuses, however this difference was not significant (Figure 35). Galactose and Gal-1P levels in GD 21 brains were significantly higher in M3/M3 fetuses than M3/WT fetuses (Figures 36, 37).

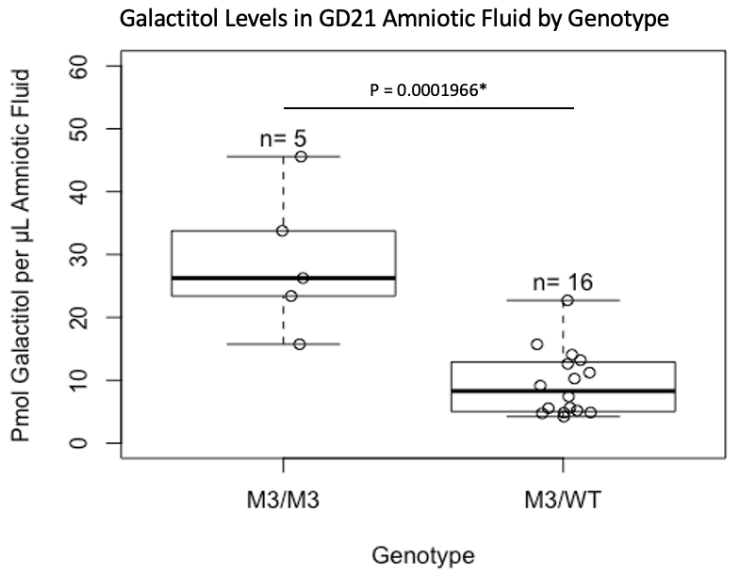


Figure 30. Galactitol Levels in GD21 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

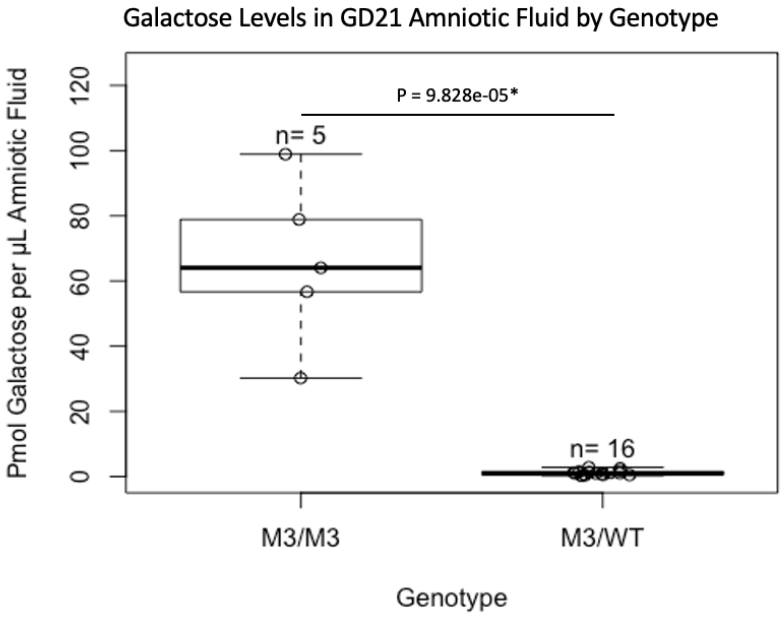


Figure 31. Galactose Levels in GD21 Amniotic Fluid by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

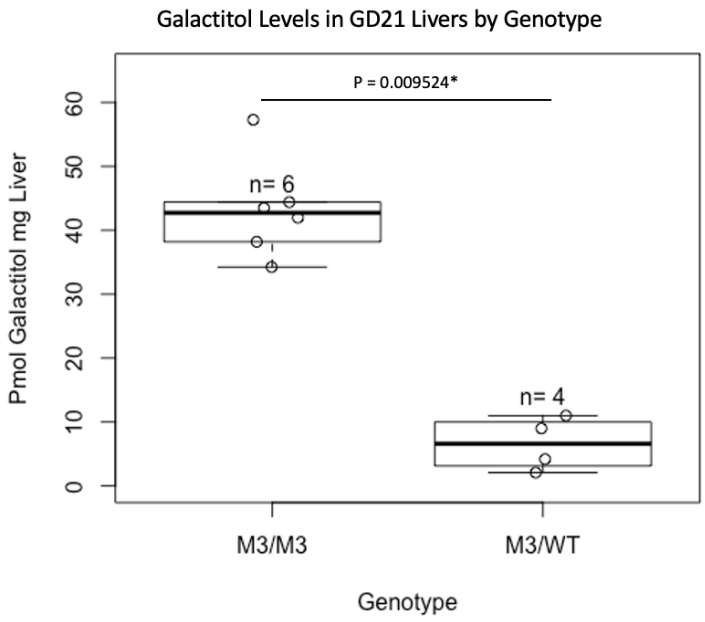


Figure 32. Galactitol Levels in GD21 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

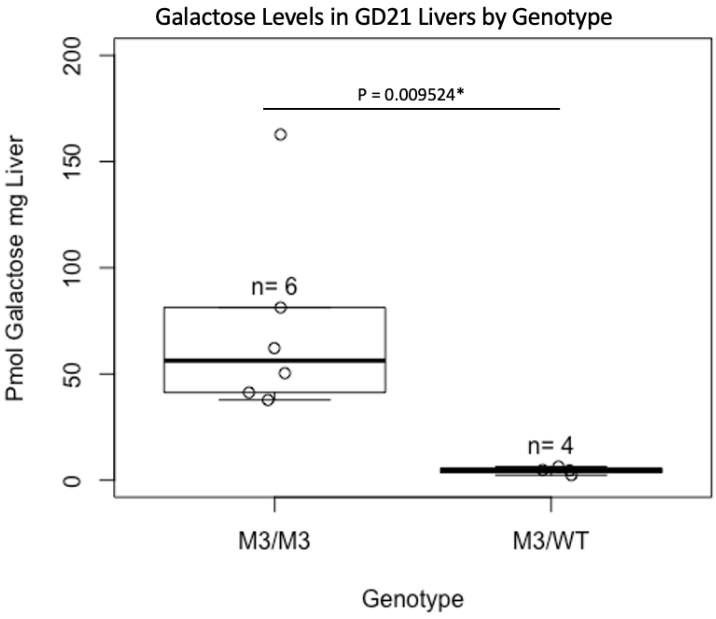


Figure 33. Galactose Levels in GD21 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

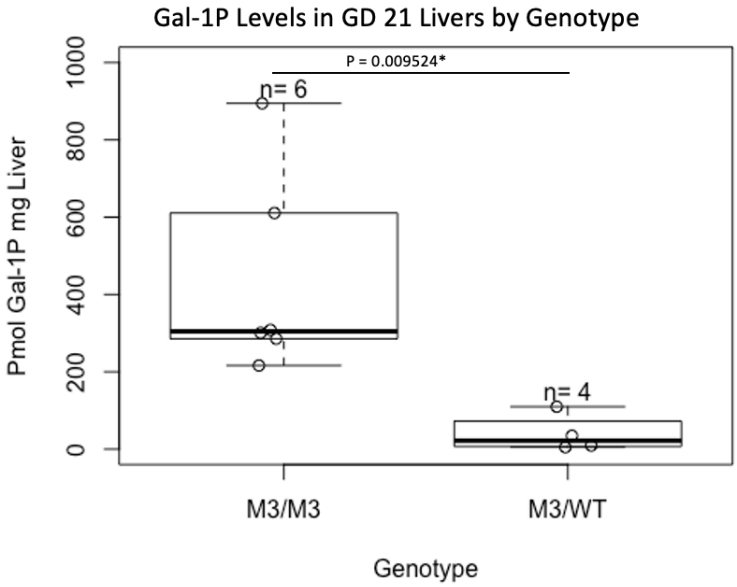


Figure 34. Gal-1P Levels in GD21 Livers by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value ($P < 0.05$).

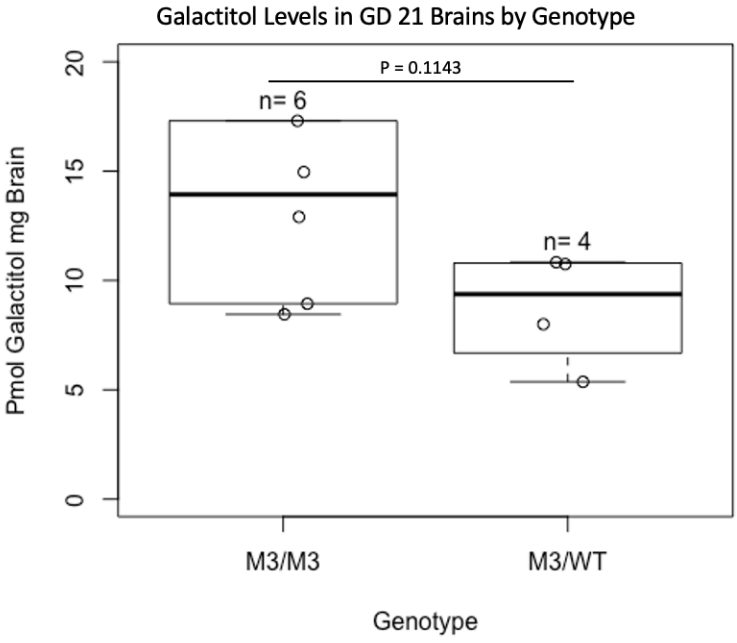


Figure 35. Galactitol Levels in GD21 Brains by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.

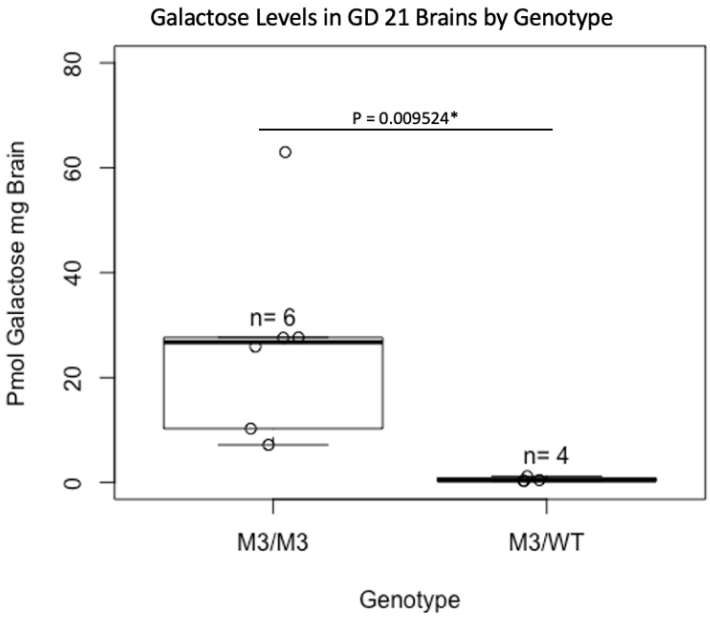


Figure 36. Galactose Levels in GD21 Brains by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

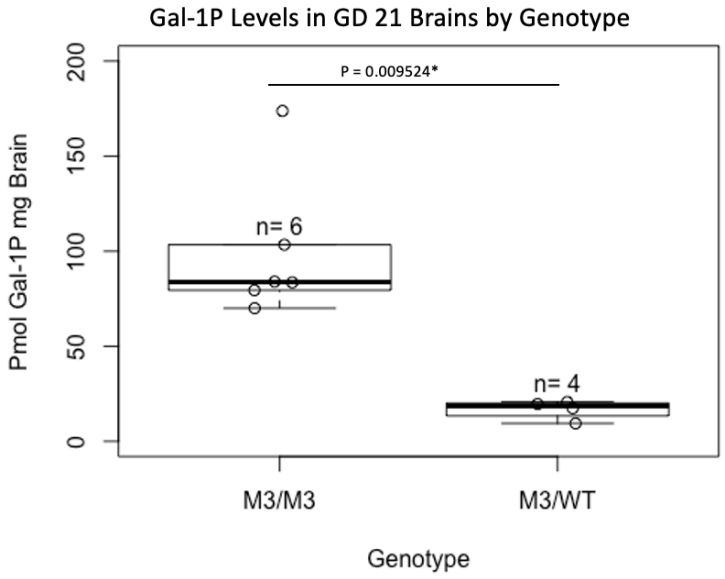


Figure 37. Gal-1P Levels in GD21 Brains by Genotype. All fetuses were from M3/WT (maternal) by M3/M3 (paternal) crosses.
*Indicates significant p-value (P < 0.05).

Discussion

As a whole, these data demonstrate that, at all points in gestation tested, metabolite levels were generally higher in M3/M3 fetuses than M3/WT controls and that increases in metabolite levels through gestation were more significant in M3/M3 fetuses. While galactitol levels in M3/WT amniotic fluid did increase from gestational days 19 to 21, galactitol levels in M3/M3 amniotic fluid increased between all timepoints except gestational days 17 and 19 (Figures 5, 6, S1). Direct comparisons between each timepoint also show that galactitol levels were significantly higher in M3/M3 amniotic fluid than M3/WT amniotic fluid at GD 15, GD 19, and GD 21 (Figures 15, 20, 25, 30). This indicates that while an increase in galactitol levels in amniotic fluid should be expected in all rat fetuses during gestation, the accumulation of galactitol was significantly more severe in M3/M3 than M3/WT amniotic fluid. While galactose levels in M3/WT amniotic fluid significantly decreased from gestational day 19 to 21, galactose levels in M3/M3 amniotic fluid remained constant (Figures 7, 8, S2). Direct comparisons between each timepoint show that galactose levels were significantly higher in M3/M3 amniotic fluid than M3/WT at GD 15 and GD 21, and trending towards being higher but not significantly different in M3/M3 amniotic fluid versus M3/WT at GD 17 and 19 (Figures 16, 21, 26, 31).

Amniotic fluid is similar to fetal plasma during the early stages of gestation before the skin keratinizes, but by the mid-to-late gestational timepoints represented by these data from GD 17 onwards the amniotic fluid largely consists of fetal urine (28, 29). Because of this changing composition of amniotic fluid, it can be considered a measure of average whole body metabolite output. The galactitol and galactose levels in M3/M3 versus M3/WT amniotic fluid

suggest that while there is a difference in a GALT-null rat's metabolites as early as GD 15, this difference becomes more severe later in development. The galactose levels in GD 21 M3/WT amniotic fluid in particular suggest a notable increase in GALT enzyme activity during this point in gestation for GALT+ rats, because the galactose levels ranged about 10-70 pmol per µL amniotic fluid for the three earliest timepoints but suddenly decreased to nearly 0 pmol per µL amniotic fluid at GD 21. GALT enzyme activity testing should be performed in the future on samples with enough remaining amniotic fluid in order to confirm this explanation for decreased galactose levels at GD 21.

While galactitol levels in M3/WT livers showed no significant changes through gestation, galactitol levels in M3/M3 livers showed a significant increase over time (Figures 9, 10, S3). Direct comparisons between each timepoint show that galactitol levels were significantly higher in M3/M3 livers than M3/WT livers at GD 15, GD 17, GD 19, and GD 21 (Figures 17, 22, 27, 32). Similar to galactitol, M3/WT livers showed no significant changes in galactose levels through gestation while M3/M3 livers showed a significant increase over time (Figures 11, 12, S4). Direct comparisons between each timepoint show that galactose levels were significantly higher in M3/M3 livers than M3/WT livers at GD 17, GD 19, and GD 21 (Figures 18, 23, 28, 33). Gal-1P levels also showed no significant changes through gestation in M3/WT livers, and no significant changes between consecutive gestational days in M3/M3 livers (Figures 13, 14, S5). Direct comparisons between each timepoint show that Gal-1P levels were significantly higher in M3/M3 livers than M3/WT livers at GD 15, GD 17, GD 19, and GD 21 (Figures 19, 24, 29, 34).

Liver failure is a common cause of death in human neonates with CG whose galactose consumption was not restricted within the first few days of life (1). The galactitol and galactose

levels in M3/M3 fetuses versus M3/WT fetuses through gestation suggest that metabolite accumulation begins in GALT-null fetuses by at least GD 15 and continues to worsen through gestation. The Gal-1P levels in M3/M3 fetuses versus M3/WT fetuses through gestation suggest that Gal-1P accumulation begins in GALT-null fetuses by at least GD 15 and fluctuates but does not worsen overall through gestation.

In GD 21 brains, galactose and Gal-1P levels were elevated in M3/M3 fetuses compared to M3/WTs while galactitol was not significantly different (Figures 35, 36, 37). A previous study suggested that elevated fetal Gal-1P levels indirectly cause the CNS phenotypes of CG by interfering with myo-inositol's interactions with neurons (12). The elevated Gal-1P levels observed in GD 21 fetal brains provides evidence to support this argument. Galactitol levels overall were very low in GD 21 brains compared to livers and amniotic fluid, so the lower overall amount may explain the lack of significant accumulation in GD 21 brains.

These elevated metabolite levels in M3/M3 fetuses compared to M3/WT fetuses demonstrate that galactose is produced endogenously in fetuses. Future comparisons of liver and brain metabolites between fetuses and nursing pups could be performed in order to determine the degree of endogenous galactose production and the difference in severity of galactose metabolite accumulation between GALT-null rat fetuses and neonates.

CHAPTER 3: PARTIAL PRENATAL METABOLIC CORRECTION OF GALT-NULL FETUSES FROM CO-GESTATING HETEROZYGOUS SIBLINGS

Introduction

The excess neonatal demise documented in M3/M3 pups from M3/M3 by M3/M3 litters but not in M3/M3 pups from crosses involving an M3/M3 mother and an M3/WT father suggests that there may be some prenatal factor contributing to the compromised viability of these neonates (22). One possible explanation for this phenomenon is that co-gestation of M3/M3 fetuses with M3/WT fetuses minimizes prenatal accumulation of the Leloir Pathway metabolites which ostensibly cause the adverse phenotypes of CG. We hypothesize that this cross-correction does occur, and that we will see lower levels of galactose, galactitol, and Gal-1P in M3/M3 fetuses from M3/M3 by M3/WT crosses, where at least some of the fetuses will have GALT activity, versus M3/M3 fetuses from M3/M3 by M3/M3 crosses, where all fetuses are GALT-null.

Methods

All methods involving live rats were performed using procedures approved by the Emory Institutional Animal Care and Use Committee (IACUC) under protocol 201700095. All rat estrus cycling and crosses, gestational tissue collections, metabolite extractions, and metabolite quantifications were performed as described in the methods section of Chapter 2. All live rats were fed LabDiet 5053, which derives 1% of its calories from galactose.

Sample Size

In consideration of the goal to determine if M3/M3 fetuses co-gestating with M3/WT siblings, the two comparison groups are M3/M3 fetuses from M3/M3 by M3/M3 crosses and M3/M3 fetuses from M3/M3 (maternal) by M3/WT crosses. M3/M3 fetuses from M3/WT (maternal) by M3/M3 (paternal) are excluded from this comparison because the heterozygous genotype of the mother would be a confounding variable. Due to HPLC time constraints and the prioritization of tasks, this comparison was only made for GD 21 fetuses. GD 21 was selected because that was the timepoint closest to birth and the timepoint in which the greatest differences in metabolite levels between M3/M3 and M3/WT fetuses were observed in Chapter 2. The following table describes the samples analyzed for each tissue.

TABLE 8. Demographics of M3/M3 sibling phenotype correction samples analyzed.

FKRC	Liver (n)	Brain (n)	Amniotic Fluid (n)	# of M3/WTs in Litter	Total Litter Size
377	5	5	5	5	15
378	4	4	5	9	15
387	4	4	4	4	4

Statistical Methods

Graphs depicting the comparison between M3/M3 fetuses with M3/M3 versus M3/WT fathers were created for each metabolite and tissue, with the x-axis representing the proportion of M3/WT fetuses within each litter. There were two M3/M3 (maternal) by M3/WT (paternal) crosses set up for this GD and each had 15 fetuses total. A bimodal distribution was

observed when these two litters were graphed within the same group on a graph showing metabolites for M3/M3 fetuses from M3/M3 mothers with M3/M3 versus M3/WT fathers, so these two litters were separated into different groups in order to explore the correction effect of M3/WT siblings based on their proportion within the litter. The Kruskal-Wallis Test was used to determine overall significance at $\alpha = 0.05$. The Kruskal-Wallis Test was used rather than an analysis of variance (ANOVA) to account for the non-normal distribution of the data. Pairwise Wilcoxon Tests were then performed with the Benjamini and Hochberg correction adjustment method applied. The Benjamini and Hochberg correction was selected because it controls the false discovery rate (27). $\alpha = 0.05$ was also used to determine the significance of each pairwise comparison.

Results

Galactitol Levels in GD 21 Fetal Tissue by Proportion of Heterozygous Siblings

Galactitol levels in M3/M3 amniotic fluid were significantly lower in fetuses from the litter with the highest proportion of heterozygotes compared to fetuses from both other litters, with a lower proportion of heterozygotes and no heterozygotes, which did not significantly differ from each other (Figure 38). Galactitol levels in M3/M3 livers were significantly lower in fetuses from both litters containing heterozygotes compared to the litter containing no heterozygotes, but not significantly different between the two litters with heterozygotes (Figure 39). Galactitol levels in the M3/M3 brains did not differ between any of the groups regardless of heterozygous siblings (Figure 40).

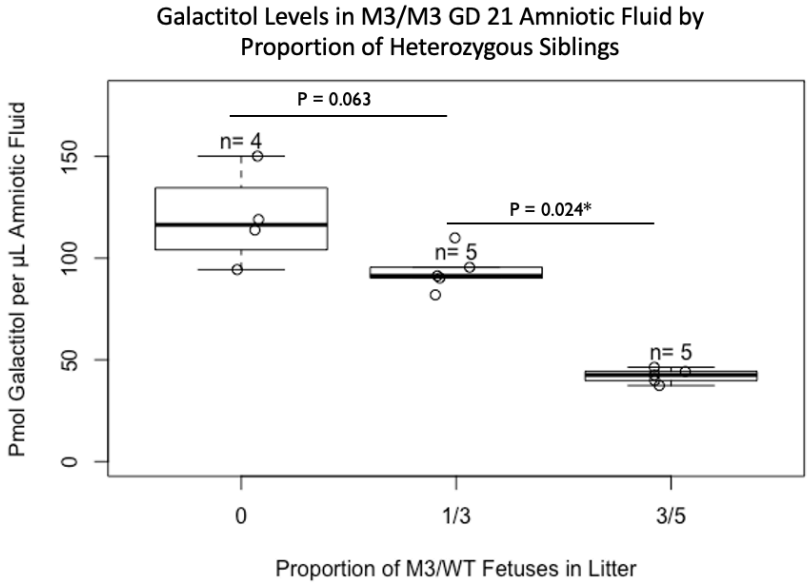


Figure 38. Galactitol Levels in GD 21 M3/M3 Amniotic Fluid by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.004879*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

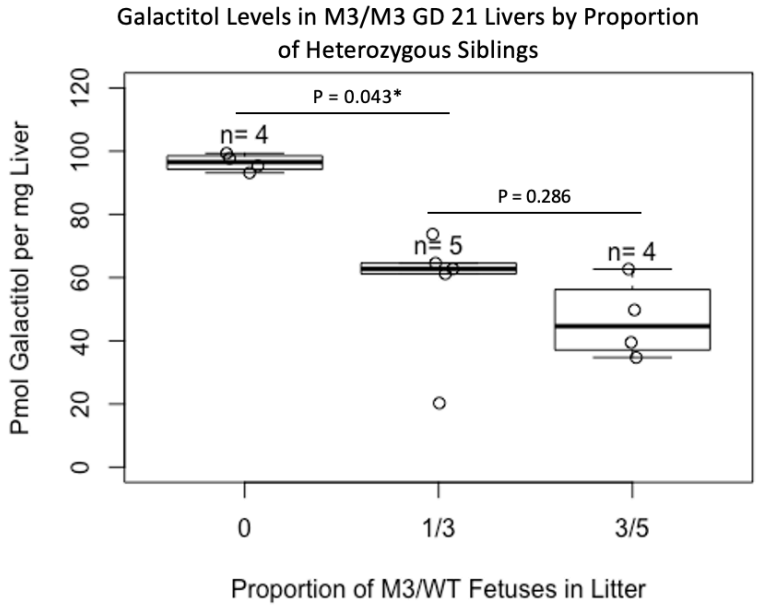


Figure 39. Galactitol Levels in GD 21 M3/M3 Livers by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.01458*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

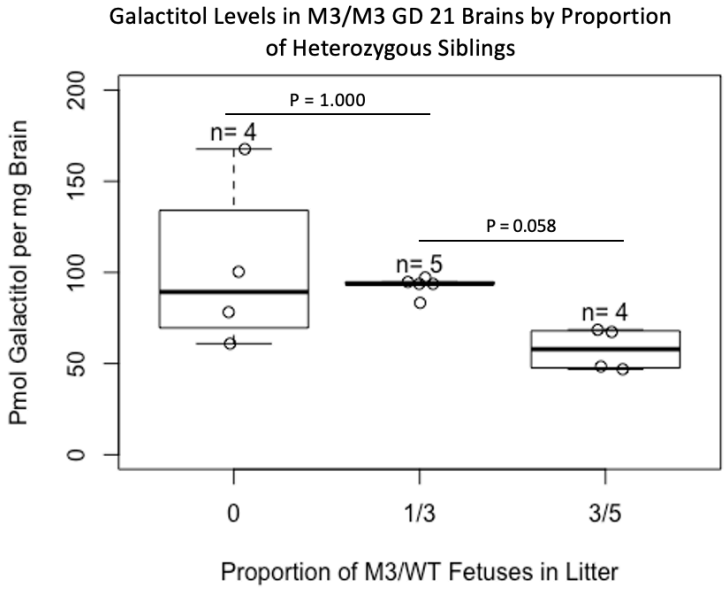


Figure 40. Galactitol Levels in GD 21 M3/M3 Brains by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.04622*, pairwise comparisons shown on graph.
*Indicates significant p-value ($P < 0.05$).

Galactose Levels in GD 21 Fetal Tissue by Proportion of Heterozygous Siblings

Galactose levels in M3/M3 amniotic fluid were significantly lower in the fetuses from the litter with the higher proportion of heterozygotes compared to fetuses from the litter with the lower proportion of heterozygotes and in the fetuses from the litter with the lower proportion of heterozygotes compared to the fetuses from the litter with no heterozygotes (Figure 41). Galactose levels in M3/M3 livers were significantly lower in fetuses from the litter with the higher proportion of heterozygotes compared to fetuses from both other litters, which did not significantly differ (Figure 42). Galactose levels in M3/M3 brains were not significantly different between any of the comparison groups (Figure 43).

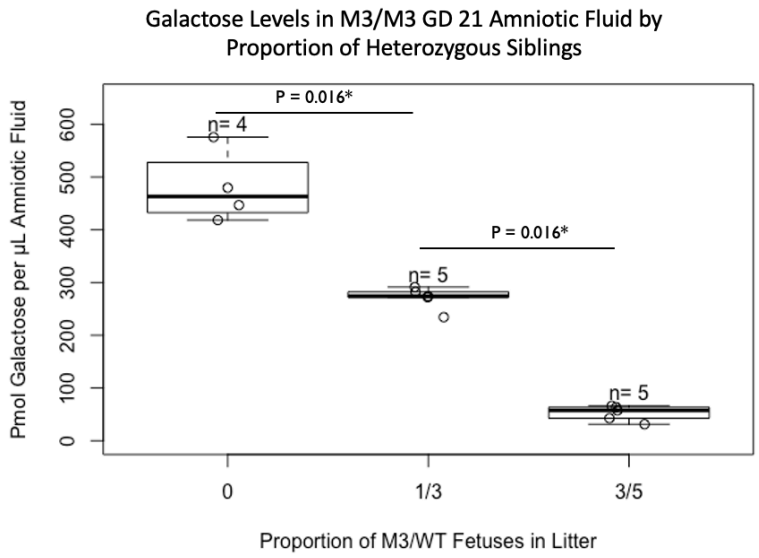


Figure 41. Galactose Levels in GD 21 M3/M3 Amniotic Fluid by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.003071*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

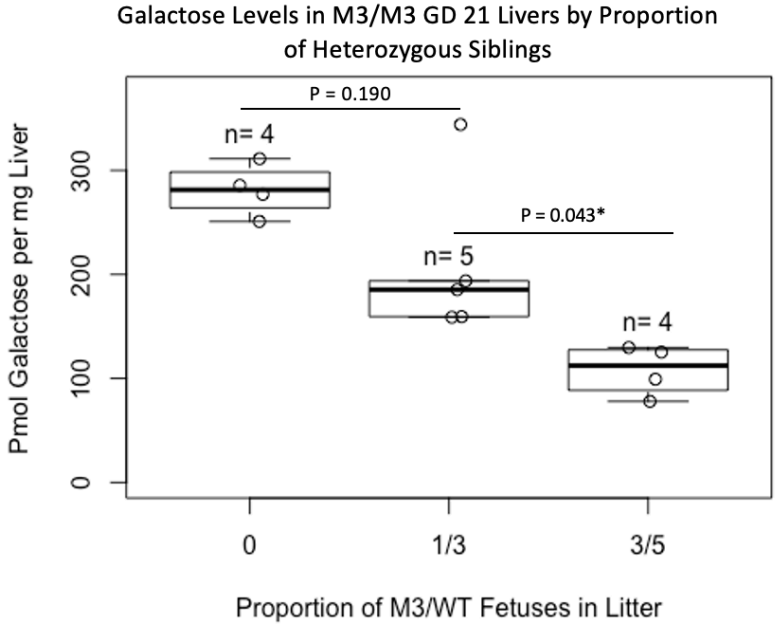


Figure 42. Galactose Levels in GD 21 M3/M3 Livers by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.01239*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

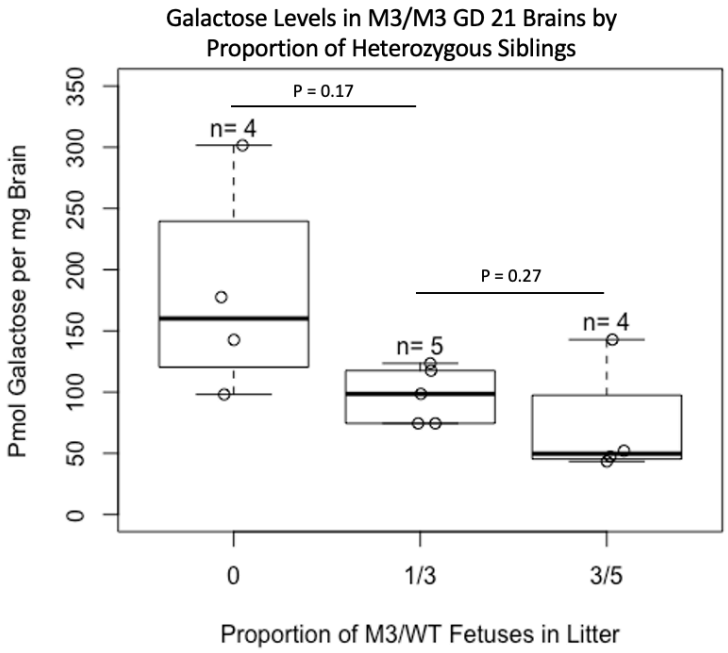


Figure 43. Galactose Levels in GD 21 M3/M3 Brains by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.08864, pairwise comparisons shown on graph.

Gal-1P Levels in GD 21 Fetal Tissue by Proportion of Heterozygous Siblings

Gal-1P levels in M3/M3 livers were significantly lower in fetuses from the litter with the higher proportion of heterozygotes than fetuses from both the litter with the lower proportion of heterozygotes and the litter with no heterozygotes, which did not significantly differ (Figure 44). Gal-1P levels in M3/M3 brains were significantly higher in fetuses from the litter with the lower proportion of heterozygotes than fetuses from either other litter, but there was no significant difference between fetuses from the litter with no heterozygotes and fetuses from the litter with a larger proportion of heterozygotes (Figure 45).

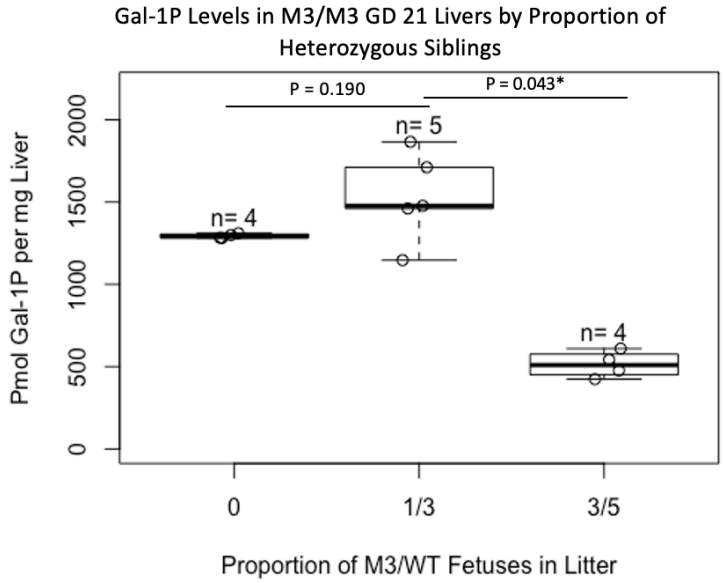


Figure 44. Gal-1P Levels in GD 21 M3/M3 Livers by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.01239*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

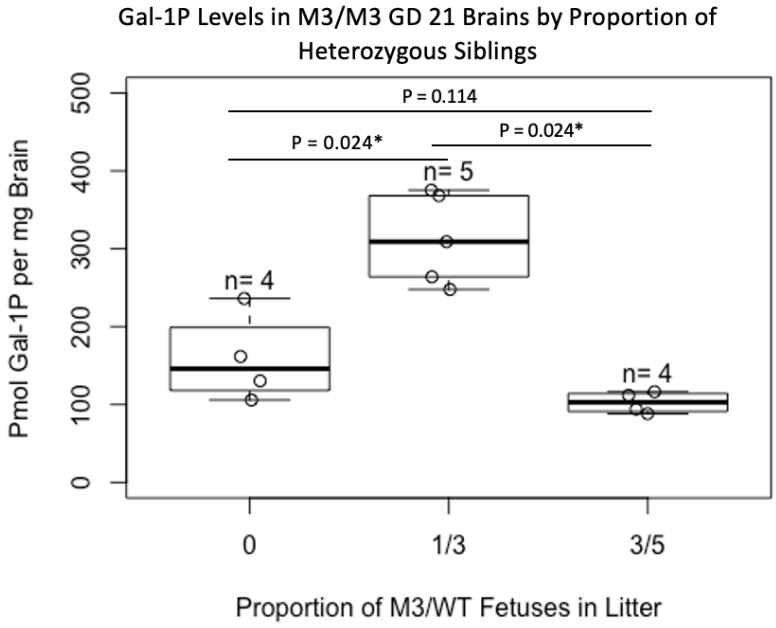


Figure 45. Gal-1P Levels in GD 21 M3/M3 Brains by Proportion of Heterozygotes in Litter. All fetuses have M3/M3 mothers. Kruskal-Wallis Test P = 0.007604*, pairwise comparisons shown on graph.
*Indicates significant p-value (P < 0.05).

Discussion

As a whole, increased proportion of heterozygous siblings within a litter seemed to have a negative correlation with metabolite levels in M3/M3 fetuses. For galactitol in GD 21 amniotic fluid, galactose in GD 21 livers, and Gal-1P in GD 21 livers, metabolite levels were significantly lower in M3/M3 fetuses from the litter with the higher proportion of heterozygotes than both other litters (Figures 38, 42, 44). For galactose in GD 21 amniotic fluid, metabolite levels were significantly lower in M3/M3 fetuses for each group of increasing M3/WT proportion (Figure 41). For galactitol in GD 21 livers, metabolite levels were significantly lower in M3/M3 fetuses from both litters containing M3/WT siblings than in the M3/M3 by M3/M3 cross (Figure 39). For galactitol and galactose in GD 21 brains, metabolite levels were not significantly different between any of the litters (Figures 40, 43). For Gal-1P levels in GD 21 brains, metabolite levels were significantly higher in M3/M3 fetuses from the litter containing the lower proportion of heterozygotes than in either other litter (Figure 45).

Other than galactitol and Gal-1P levels in GD 21 brains, these data all show a general trend towards increased cross-correction of metabolite accumulation with increased proportion of heterozygotes present. Galactitol and galactose are substances that can pass through semipermeable lipid bilayers, such as those found in cell members (30). Gal-1P, on the other hand, is considered an intracellular metabolite because it cannot pass through cell membranes (26). It makes sense, therefore, to see stronger trends in decreased amount of galactitol and galactose because those metabolites can diffuse from M3/M3 fetuses into M3/WT neighbors. The lower levels of Gal-1P that were observed in M3/M3 livers from fetuses

with the highest proportion of heterozygous siblings were likely caused by the downstream effects of galactose diffusing into M3/WT neighbors then going through the Leloir Pathway or being converted into Gal-1P within the M3/WTs. These results do support the hypothesis that there is a cross-correction among fetuses gestating together which causes M3/M3 fetuses from M3/M3 by M3/WT crosses to have less severe prenatal metabolite accumulation than M3/M3 fetuses from M3/M3 by M3/M3 crosses, which may contribute to the excess neonatal demise observed in M3/M3 by M3/M3 crosses only.

CHAPTER 4: PARTIAL PRENATAL METABOLIC CORRECTION OF GALT-NULL FETUSES FROM GESTATION WITHIN A HETEROZYGOUS MOTHER

Introduction

The excess neonatal demise of M3/M3 pups from M3/M3 by M3/M3 crosses only suggests that some prenatal or perinatal factor impacts the viability of these pups (22). Although high rates of neonatal demise were not recorded in M3/M3 (maternal) by M3/WT (paternal) litters, the effect of the mother's genotype on prenatal metabolite accumulation should still be considered (22). If having a heterozygous mom does decrease the severity of metabolite accumulation in GALT-null fetuses, then M3/M3 fetuses from M3/WT (maternal) by M3/M3 (paternal) crosses should have lower levels of galactose, galactitol, and Gal-1P than M3/M3 fetuses from M3/M3 (maternal) by M3/WT (paternal) crosses. If this were the case, it would ostensibly be because GALT activity in the mother's body will minimize accumulation of galactose metabolites than can cross from the fetal bloodstream to the maternal bloodstream at the placenta.

Methods

All methods involving live rats were performed using procedures approved by the Emory Institutional Animal Care and Use Committee (IACUC) under protocol 201700095. All rat estrus cycling and crosses, gestational tissue collections, metabolite extractions, and metabolite quantifications were performed as described in the methods section of Chapter 2. All live rats were fed LabDiet 5053, which derives 1% of its calories from galactose.

Sample Size

In consideration of the goal of determining if maternal genotype affects the prenatal metabolites of GALT-null fetuses, the comparison groups are M3/M3 fetuses from M3/M3 (maternal) by M3/WT (paternal) crosses and M3/M3 fetuses from M3/WT (maternal) by M3/M3 (paternal) crosses. M3/M3 fetuses from M3/M3 by M3/M3 crosses were excluded from this comparison to limit the confounding factor of the proportion of M3/WT fetuses per litter. Based on the results from Chapter 3, only two litters of each type were compared to limit confounding from proportion of heterozygous siblings in each litter. The M3/M3 (maternal) by M3/WT (paternal) litter consisted of 9 M3/WT fetuses and 6 M3/M3 fetuses, giving a proportion of heterozygotes of 0.6. The M3/WT (maternal) by M3/M3 (paternal) litter consisted of 8 M3/WT fetuses and 6 M3/M3 fetuses, giving a proportion of heterozygotes of 0.5714. Due to HPLC time constraints and prioritization of tasks, these comparisons were only made for GD 21 fetuses. GD 21 was selected because that is the timepoint closest to birth as well as the timepoint during which the greatest differences in metabolite levels between M3/M3 and M3/WT fetuses was observed in Chapter 2. The following tables describe the samples analyzed for each tissue.

TABLE 9. Demographics of M3/M3 maternal phenotype correction samples analyzed.

Maternal Genotype	Liver (n)	Brain (n)	Amniotic Fluid (n)	# of M3/WTs in Litter	Total Litter Size
M3/M3	4	4	5	9	15
M3/WT	4	4	4	8	14

Statistical Methods

Graphs depicting the comparison between M3/M3 and M3/WT fetuses with M3/M3 versus M3/WT mothers were created for each metabolite and tissue, and a Wilcoxon rank sum test for each comparison to determine statistical significance using $\alpha = 0.05$. The Wilcoxon rank sum test was selected instead of a t-test to account for the non-normal distribution of the data.

Results

Galactitol Levels in M3/M3 GD 21 Fetal Tissues by Maternal Genotype

Galactitol levels were significantly higher in M3/M3 GD 21 amniotic fluid from M3/M3 (maternal) by M3/WT (paternal) crosses than M3/WT (maternal) by M3/M3 (paternal) crosses (Figure 46). Galactitol levels in M3/M3 GD 21 livers did not significantly differ by maternal genotype (Figure 47). Galactitol levels in GD 21 brains were significantly higher in M3/M3 (maternal) by M3/WT (paternal) than M3/WT (maternal) by M3/M3 (paternal) crosses (Figure 48).

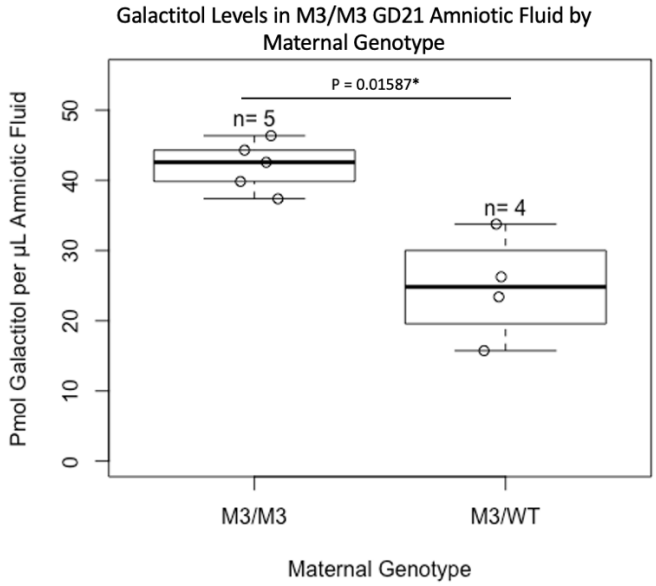


Figure 46. Galactitol Levels in M3/M3 GD21 Amniotic Fluid by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.
*Indicates significant p-value (P < 0.05).

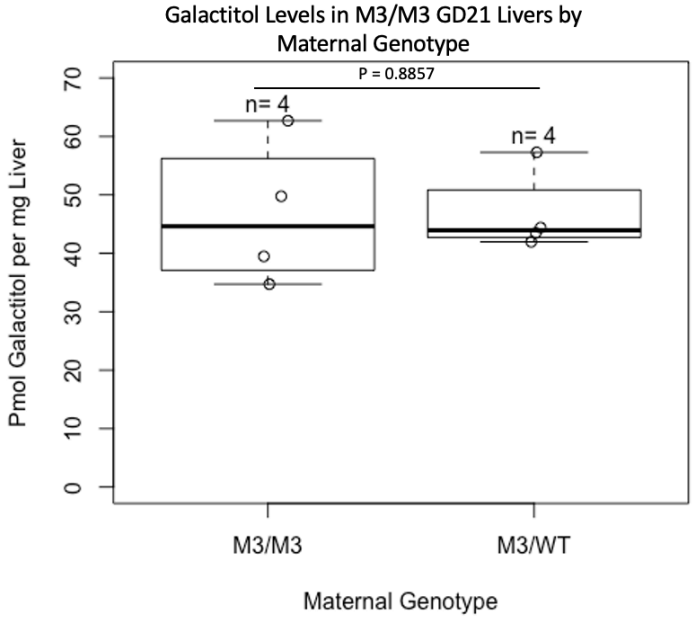


Figure 47. Galactitol Levels in M3/M3 GD21 Livers by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

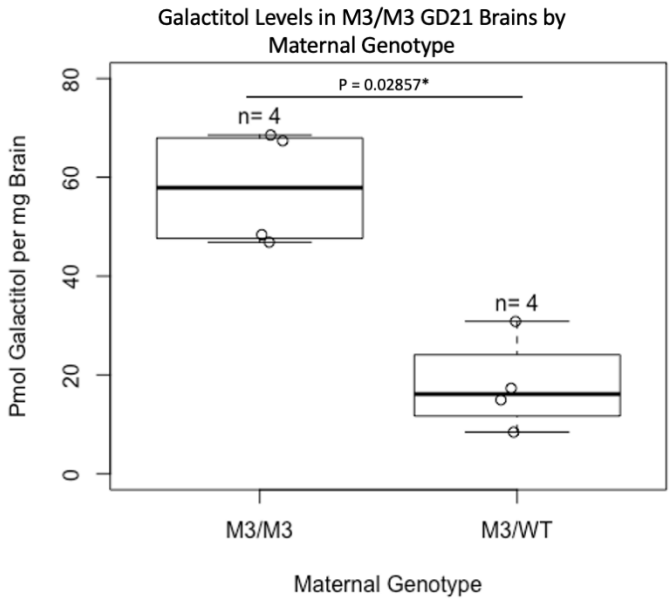


Figure 48. Galactitol Levels in M3/M3 GD21 Brains by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.
*Indicates significant p-value ($P < 0.05$).

Galactose Levels in M3/M3 GD 21 Fetal Tissue by Maternal Genotype

Galactose levels in M3/M3 GD 21 fetal tissues did not vary significantly by maternal genotype in amniotic fluid, livers, or brains (Figures 49, 50, 51).

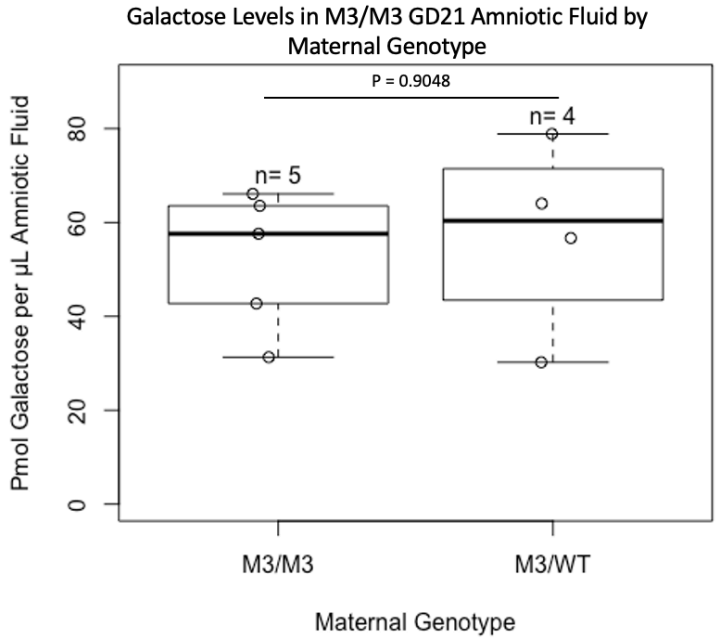


Figure 49. Galactose Levels in M3/M3 GD21 Amniotic Fluid by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

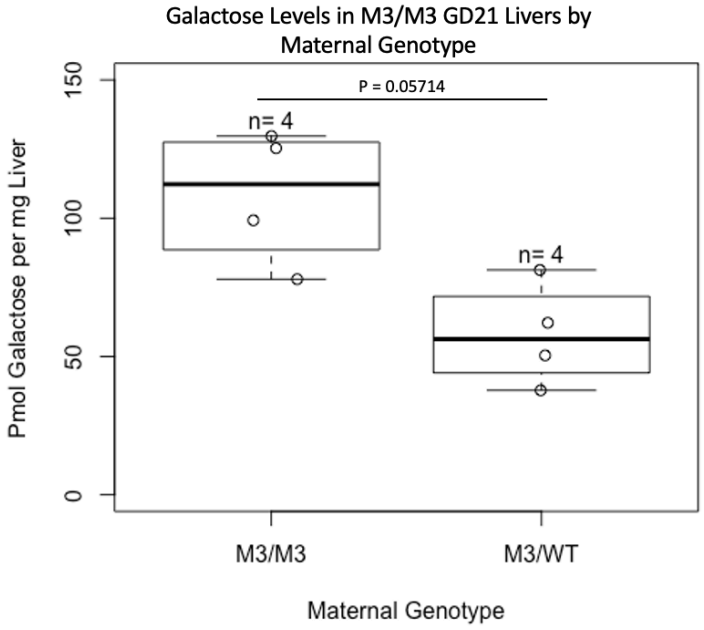


Figure 50. Galactose Levels in M3/M3 GD21 Livers by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

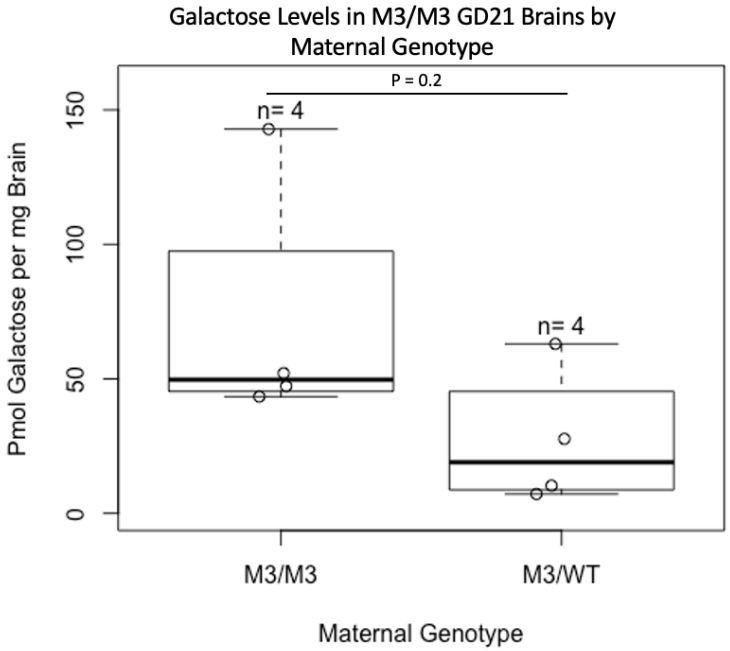


Figure 51. Galactose Levels in M3/M3 GD21 Brains by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

Gal-1P Levels in M3/M3 GD 21 Fetal Tissue by Maternal Genotype

Gal-1P levels in M3/M3 GD 21 fetal tissues did not vary significantly by maternal genotype in livers or brains (Figures 52, 53).

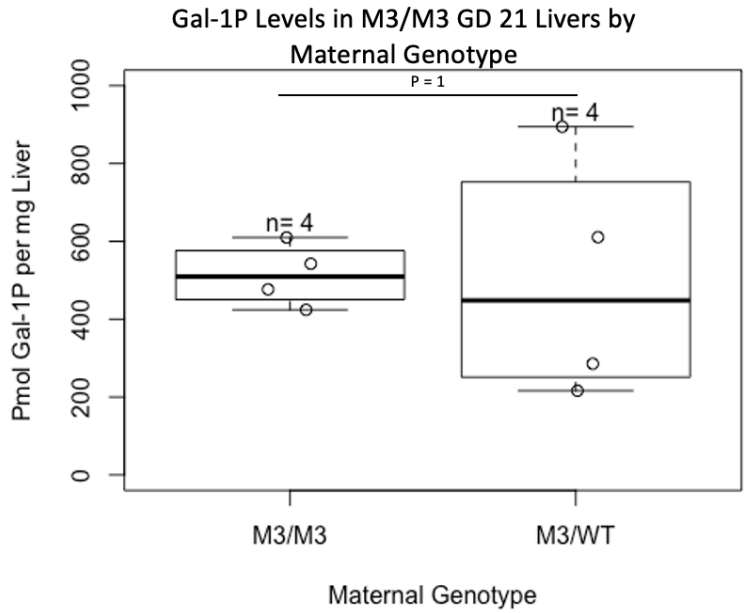


Figure 52. Gal-1P Levels in M3/M3 GD21 Livers by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

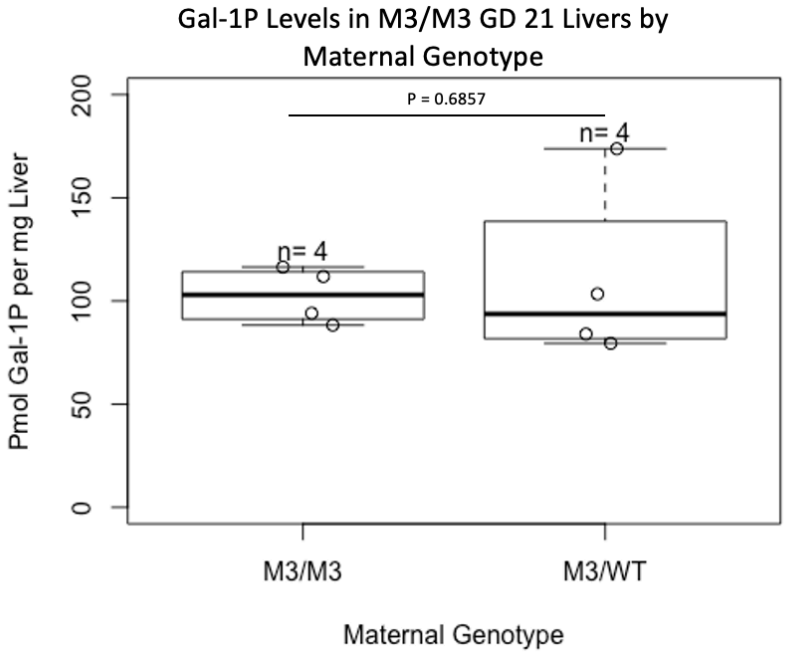


Figure 53. Gal-1P Levels in M3/M3 GD21 Brains by Maternal Genotype. All fetuses were from litters containing 60% or 57.14% M3/WTs.

Discussion

With the exception of galactitol levels in amniotic fluid and brains, maternal genotype did not seem to significantly affect the metabolite accumulation of M3/M3 fetuses through gestation. Even though the increased pre-weaning neonatal demise being observed only in M3/M3 by M3/M3 crosses and not in M3/M3 by M3/WT crosses regardless of maternal genotype suggested that sibling genotype might be a more important factor for prenatal metabolite correction, this is still a somewhat surprising result (22). The rat placenta is a major interface for exchange between fetal and maternal tissue, and the large surface area of the placenta causes it to come into contact with relatively large amounts of maternal blood (31). Like in humans, the rat umbilical cord which connects the fetus to the placenta contains two arteries and a vein (32). It would make sense for M3/WT maternal blood to contain lower galactose metabolite levels and thus deposit fewer metabolites into the fetuses through the placenta than M3/M3 rats, then also fetal metabolites could return to maternal circulation through the placenta and be metabolized by maternal enzymes.

One reason why maternal genotype may not have as much of an impact on fetal metabolite accumulation as expected would be that each litter contains around 4-16 fetuses from only one dam. If endogenous production of galactose is happening within the dam as well as all the fetuses, then galactose metabolite levels may rise enough that maternal GALT activity is too low to significantly correct metabolite accumulation. Another possible explanation for the limited impact of maternal genotype on fetal metabolite accumulation would be the effect of the heterozygous siblings. As demonstrated by the data from Chapter 3, increased proportions

of heterozygous siblings do generally correlate with lower levels of galactose metabolites in M3/M3 fetuses. Since the litters compared to examine the impact of maternal genotype contain both M3/WT and M3/M3 fetuses, perhaps sibling cross-correction is so impactful that maternal protection is less essential. Regardless of the reason for it, the lack of an impact that maternal genotype has on fetal metabolite accumulation supports the suggestion that increased neonatal demise in M3/M3 by M3/M3 crosses results from prenatal metabolite accumulation which is prevented in M3/M3 by M3/WT crosses in both directions by correction from co-gestating siblings.

CHAPTER 5: GENERAL DISCUSSION

Discussion

CG is an autosomal recessive disorder characterized by a profound deficiency of the GALT enzyme, and the only current treatment available is implementing a galactose-restricted diet (1). Previous studies suggested that prenatal galactose exposure is responsible for the ovarian insufficiency phenotype of CG (9, 10, 11). Another study reported increased Gal-1P and galactitol levels in galactosemia cord red blood cells and amniotic fluid, suggesting that myo-inositol deficiency caused by accumulation of these metabolites may cause the CNS phenotypes associated with CG (12). The first aim of this study was to gain a better general understanding of changes in metabolites through gestation of GALT-null rat fetuses. In general, galactose metabolite levels were higher in M3/M3 fetuses than M3/WT at all four gestational days measured and these already elevated levels increased over time. This suggests that metabolite accumulation contributing to the complications associated with CG starts by GD 15 in rats and worsens over time. Since the average gestational length in rats is 21-23 days (21), GD 15 in rats would correspond to about the start of the third trimester in humans by proportion of days. This metabolite accumulation may likely begin prior to GD 15, however younger gestational ages were outside of the scope of this project due to overall fetus size being so small that metabolite extractions would need to involve the entire fetus rather than specific tissues. These findings have implications for development of future treatments for CG, because any treatment started postnatally would need to reverse the prenatal damage as well as prevent future damage. For this reason, it would be optimal to develop prenatal treatments for CG.

Another aim of this study was to determine the cause of excess neonatal demise observed in M3/M3 rats from M3/M3 by M3/M3 crosses. While about 40% of GALT-null pups from 34 M3/M3 by M3/M3 crosses survived to weaning, 90% of GALT-null pups from 30 M3/M3 by M3/WT crosses (both directions) survived to weaning (22). The first possible explanation for this explored was the possibility of cross-correction of M3/M3 fetal metabolites by co-gestating M3/WT siblings. In general, the results from these comparisons showed that higher proportions of heterozygotes within litters corresponded with lower metabolite levels in M3/M3 fetuses. This supports the hypothesis of cross-correction of M3/M3 metabolites by co-gestation with M3/WT siblings. Another possible contribution to this observation is correction from gestating within M3/WT mothers. While this alone could not explain the neonatal demise observed because of the lack of neonatal demise in M3/M3 (maternal) by M3/WT (paternal) crosses, lower galactitol levels observed in M3/M3 fetuses from M3/WT mothers suggest that maternal genotype may have some effect. Differences in M3/M3 fetal metabolite accumulation based on maternal genotype, however, were not found in other galactose metabolites. Thus, while M3/WT maternal genotype may have a somewhat protective effect on prenatal galactitol accumulation in M3/M3 fetuses, prevention of metabolite accumulation by co-gestating M3/WT siblings likely plays a larger role in preventing the neonatal demise observed in M3/M3 by M3/M3 litters.

Limitations

One of the most important limitations in this project was the small number of samples analyzed for each group. While n=4 is a large enough sample size to perform statistical testing on, larger sample sizes would allow for much better comparisons and minimization of the effect of outliers on the analysis. Fortunately, this is a limitation that is in the process of being remedied as we are working to create more samples with larger n values representative of all comparison groups.

Another major limitation is the random litter sizes and distribution of fetal genotypes. Although a longitudinal study would theoretically be the best way of providing an overview of metabolite levels through gestation in the most controlled way possible, that is not possible when looking at fetal tissues because these tissues can only be collected after euthanasia. The sizes of gestational litters collected ranged from 4 to 15 each, as is expected when working with rats. In an ideal situation, the impact of maternal genotype on metabolite accumulation in GALT-null fetuses from Chapter 4 would be explored in litters of exactly the same size and exactly the same proportion of heterozygotes, but it would be very unlikely for that to actually occur so this comparison was made between litters of similar sizes with similar proportions of heterozygotes.

Putting These Results into Perspective and Future Directions

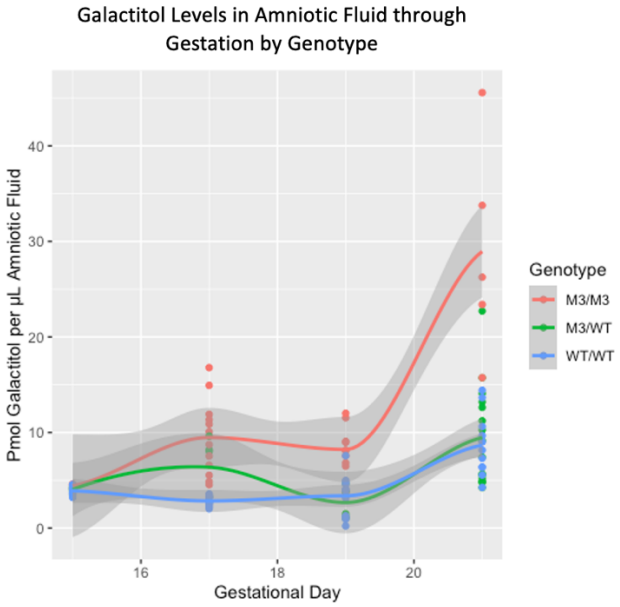
The data presented in this thesis represents only a subset of the work done to collect data for a larger ongoing project, which will have larger sample sizes and more comparison groups. Metabolite extractions have been performed on most of the samples listed in Table 4,

however metabolite quantifications have not yet been performed for all these samples due to limited HPLC resources being shared between multiple projects. While this project's scope is much more narrow than the big-picture plans for the experiment, its results provide a promising indication of future findings.

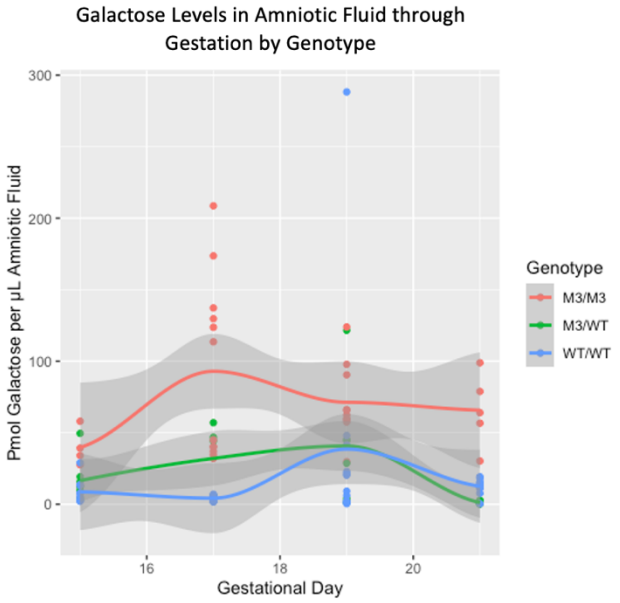
Possible areas for expansion of this project that are being considered include changing maternal galactose consumption and measuring GALT activity. Creating maternal galactose restricted and galactose exposed groups for comparison could help discover whether maternal diet during pregnancy may have any negative effects on developing fetus's metabolites. Measuring GALT activity would allow more definitive conclusions to be drawn about the relationship between metabolite levels and GALT activity. One major consideration with the potential for GALT activity assays, however, is that the entirety of the liver and brain from GD 15 and GD 17 fetuses were used for metabolite extractions, so this could only be performed for fetuses from GD 19 and GD 21 unless more samples were generated or mixed tissue samples were analyzed.

One potential area for application of this project would be in design of a treatment for CG. Since these findings demonstrate prenatal accumulation of galactose metabolites, a treatment that targets fetuses during gestation would likely be most effective. Any treatments administered later would need to not only prevent future harm but also reverse the previous damage caused by prenatal metabolite accumulation.

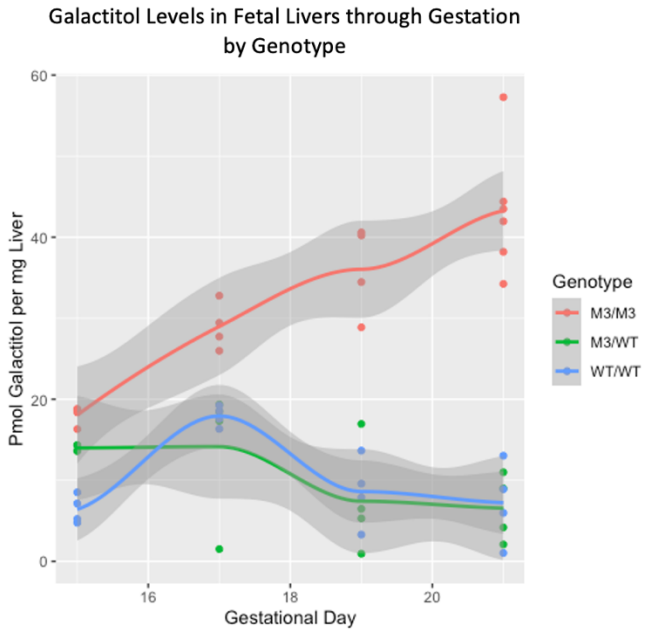
APPENDIX



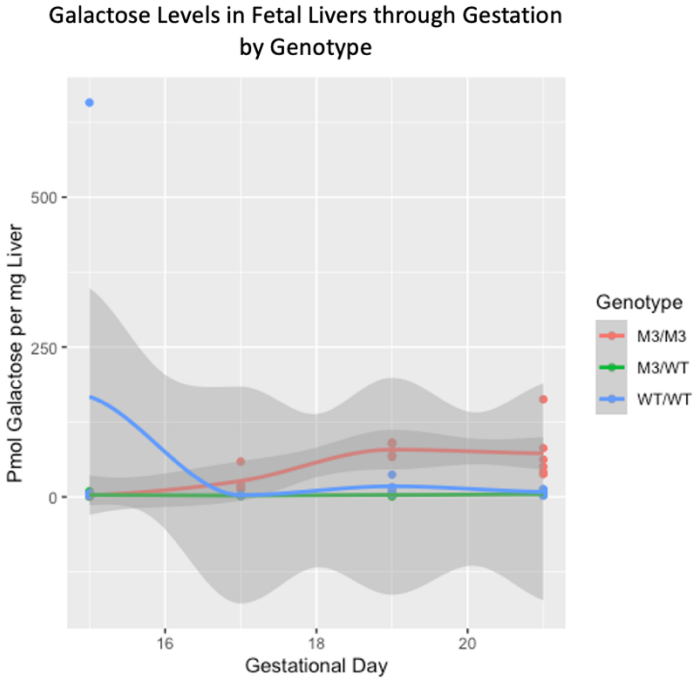
Supplemental Figure 1. Galactitol Levels in Amniotic Fluid through Gestation by Genotype. M3/M3 and M3/WT fetuses are from M3/WT (maternal) by M3/M3 (paternal) crosses, and WT/WT fetuses from WT/WT by WT/WT crosses are included as a comparison group.



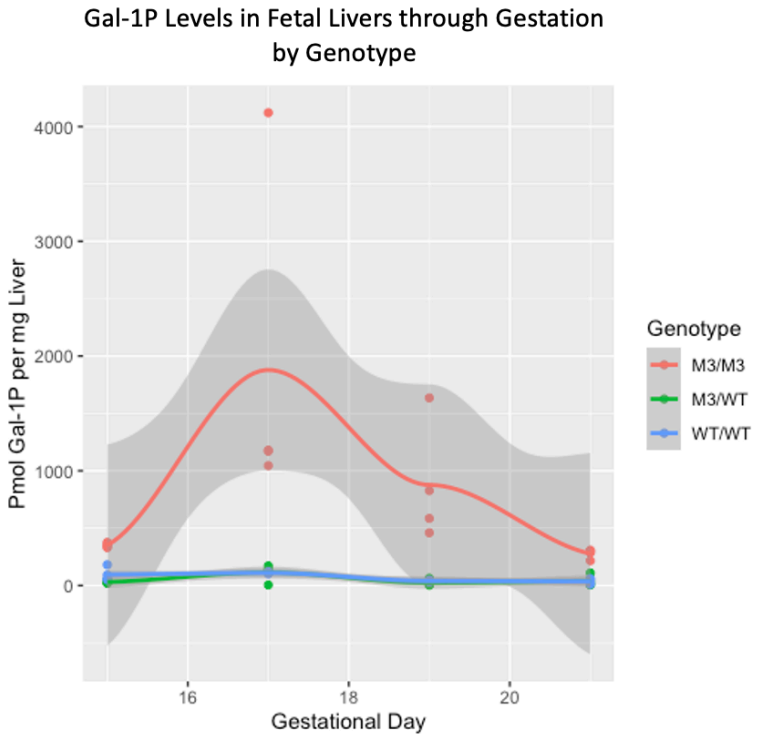
Supplemental Figure 2. Galactose Levels in Amniotic Fluid through Gestation by Genotype. M3/M3 and M3/WT fetuses are from M3/WT (maternal) by M3/M3 (paternal) crosses, and WT/WT fetuses from WT/WT by WT/WT crosses are included as a comparison group.



Supplemental Figure 3. Galactitol Levels in Fetal Livers through Gestation by Genotype. M3/M3 and M3/WT fetuses are from M3/WT (maternal) by M3/M3 (paternal) crosses, and WT/WT fetuses from WT/WT by WT/WT crosses are included as a comparison group.



Supplemental Figure 4. Galactose Levels in Fetal Livers through Gestation by Genotype. M3/M3 and M3/WT fetuses are from M3/WT (maternal) by M3/M3 (paternal) crosses, and WT/WT fetuses from WT/WT by WT/WT crosses are included as a comparison group.



Supplemental Figure 5. Gal-1P Levels in Fetal Livers through Gestation by Genotype. M3/M3 and M3/WT fetuses are from M3/WT (maternal) by M3/M3 (paternal) crosses, and WT/WT fetuses from WT/WT by WT/WT crosses are included as a comparison group.

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