THE DEVELOPMENT OF A MOUSE MODEL TO DETERMINE THE RELATION BETWEEN ASPARAGINASE-INDUCED PANCREATITIS AND GENETIC RISK FACTORS

by

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Dedication

This thesis is dedicated to all cancer fighters and survivors around the world, specifically the ones in my personal life; my grandfather who tragically passed away with throat cancer in 2015; my brother's friend Jeffery, who miraculously beat the odds of brain cancer at the age of 13 and is now studying at Dalhousie; my grandmother, who is a lymphoma survivor. Your courage, strength, and perserverance inspire me, and the fight for your health continues to push me forward in my research.

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Abstract

Acute lymphoblastic leukemia is the most common childhood cancer. Treatment protocols include the essential biologic drug asparaginase, which has helped to dramatically improve survival rates. Unfortunately, asparaginase causes several adverse drug reactions (ADRs) including pancreatitis, which afflicts up to 18% of children. Pancreatitis can be life-threatening and severe cases can result in the retraction of crucial asparaginase treatment, which can lead to a greater risk of cancer relapse. The reason why asparaginase-induced pancreatitis (AIP) develops remains unclear, but it needs to be understood to optimize the therapeutic management of individuals at a higher risk for pancreatitis development.

This thesis work was conducted in the ongoing effort to understand AIP pathogenesis. Preliminary clinical data suggested that AIP risk and/or development may be related to genetic variants of *PITX2* and *RARβ*. The main objective was to establish an AIP mouse model to investigate the functional roles of *PITX2* and *RARβ* in AIP development. In the first experiment, three mice strains, BALB/cByJ, A/J, and C57BL/6J, were intraperitoneally injected with either control PBS or 1.5 IU/g of pegylated asparaginase (peg-asp) and dissected on Day 5 post-injection for evaluation of pancreatitis development. Histological evidence suggestive of pancreatitis including mild edema, inflammatory infiltrate, fat necrosis, and islet hyperplasia were observed in pancreatic samples of some but not all peg-asp-treated mice. This suggests that some mice may have reached the AIP disease threshold but were in a state of recovery when samples were analysed. A/J mice had the most severe reaction to peg-asp via excessive weight loss and hepatotoxicity, another ADR category of asparaginase that is often linked to pancreatitis. Thus, A/J mice were chosen for a second experiment in which additional doses (0.750 or 0.375 IU/g peg-asp) and endpoints (Day 3 or 5) were evaluated. Histological signs of AIP were absent when mice were treated with the lower peg-asp doses, while some of their livers demonstrated mild toxicity. Peg-asp-treated mice serum amylase and lipase activity levels remained statistically the same as controls. No changes were observed in *PITX2* and *RARβ* mRNA expression.

Although an AIP mouse model was not successfully developed, novel insights were made that will propel the next wave of research. For the first time it was shown that different strains of mice, BALB/cByJ, A/J, and C57BL/6J, react differently to asparaginase via different degrees of weight loss and hepatotoxicity. This further solidified the idea that genetic differences are likely at play in producing variable asparaginase-induced ADRs. The role of *PITX2* and *RARβ* in AIP development requires further investigation as it was not explored at the protein level and may be influenced by other factors not identified in this study. To further optimize the AIP mouse model, earlier endpoints and multiple asparaginase injections, intramuscular or intravenous administration routes, and diet interventions such as vitamin A supplementation or depletion will be explored.

List of Abbreviations and Symbols Used

AAR	Amino acid response
ADR(s)	Adverse drug reaction(s)
AIP	Asparaginase-induced pancreatitis
ALL	Acute lymphoblastic/lymphocytic leukemia
AML	Acute myeloid leukemia
ASNS	Asparagine synthetase
BALB/c	BALB/cByJ
Ca^{2+}	Calcium
CPA2	Carboxypeptidase A2
CLL	Chronic lymphocytic leukemia
CML	Chronic myelogenous leukemia
CNS	Central nervous system
CPNDS	Canadian Pharmacogenomics Network for Drug Safety
C57	C57BL/6J
GCN2	General control nonderepressible 2
H&E	Hematoxylin and eosin
HEP	Humane endpoint
IM	Intramuscular
IP	Intraperitoneal(ly)
IV	Intravenous
PBS	Phosphate buffer saline
PEG	Polyethylene glycol

Peg-asp	Pegylated-asparaginase
Ph	Philadelphia chromosome
PITX2	Paired-like homeodomain 2
RARα	Retinoic acid receptor alpha
RARβ	Retinoic acid receptor beta
RGS6	Regulator of G-protein signaling 6
RNA poly II	RNA polymerase II
SC	Subcutaneous
SNP	Single nucleotide polymorphism(s)
ULK2	Unc-51-like kinase 2
WBC	White blood cell(s)
ΔGcn2	General control nonderepressible 2-deleted

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CHAPTER I: INTRODUCTION

1.1.0 The purpose of this thesis

1.1.1 Protecting childhood

Childhood, defined biologically as the state between infancy and puberty, is a relatively short time period that plays a large role in shaping an individual's personality and future. In the absence of genetic complications, basic life skills are acquired such as the ability to walk, verbally and socially communicate, and eat solid foods[1,2]. In the presence of education, children learn to read, write, and understand the basic strategies of essential mathematics required for functioning in daily life. Outside of the classroom, children learn to connect with peers through chatting, playing, video gaming, and texting. However, many of these fundamental essential skills and privileges that build the character and challenge the abilities of a person in a healthy, constructive way, are often lost when a child is diagnosed with the heart-wrenching disease known as cancer. Instead of playing with their parents at a playground, they may be confined to a hospital bed for months. Instead of eating ice cream in the hot summer sun with friends at the beach, they may be hooked up to an intravenous (IV) regimen, receiving multiple chemotherapies aimed to save their life. Instead of being filled with seemingly endless energy and joyful mobility, they may be too lethargic and weak to walk more than a few steps at a time.

Unfortunately, cancer robs thousands of children of their normal childhood every year across the world, reducing their overall quality of life, and often resulting in morbidity[3]. Cancer research over the past several decades has improved the survivorship of individuals with childhood cancer; however, much work is still needed to not only improve the quantity of life, but also the quality[3,4]. In the area of acute lymphoblastic or lymphocytic leukemia (ALL)

specifically, which is the most prevalent childhood cancer accounting for approximately 25% of all childhood cancer cases, the survival rate has increased dramatically from around 10% to 90% since the 1960-70s[5–9]. Clearly, there has been an applaudable enhancement in treating children with ALL, but nonetheless, long-term side effects from chemotherapies such as the increased risk of endocrine dysfunction, cardiovascular disease, neuropsychological impairment, osteonecrosis, and the development of other cancers remain an issue[10,11]. Even more so, acute side effects while actively taking therapeutic treatment, such as the development of life-threatening pancreatitis, remain of vital concern, as these adverse events pose risk to each patient's overall wellbeing and immediate survival, and can make them more susceptible to cancer relapse in the event of having to cease drug intake[12]. Thus, the underlying causes of adverse drug reactions (ADRs) are necessary to explore in order to develop mitigation methods, and to ultimately help protect the childhood of these precious patients (**Figure 1**).

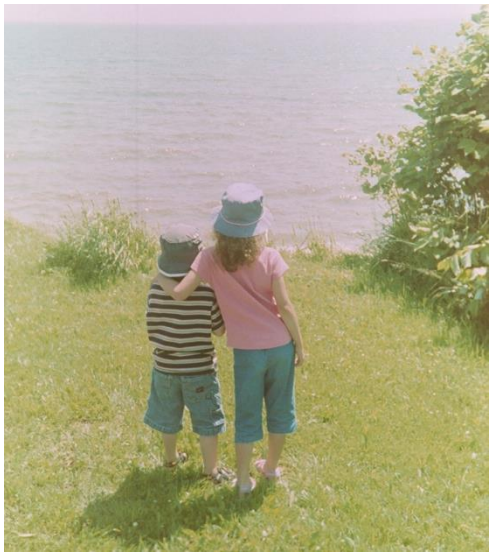

Figure 1: Preserving the Joys of Childhood

Children of all ages should ideally enjoy a healthy, playful childhood. When affected by cancer and cancer treatment ADRs, the ultimate goal should be to assist children in returning to a thriving life as soon as possible, without long-term side effects. Pictured are the author and her younger brother enjoying the oceanview as children in Eastern Passage, NS, Canada, circa 2004.

1.2.0 Acute lymphoblastic leukemia (ALL)

1.2.1 The early history of leukemia

Cancer is a generalized term that encapsulates the state of any cell in the body multiplying uncontrollably, which may also have the ability to translocate or metastasize to other regions of the body in an unhealthy, invasive manner[13]. Cancer can be very painful and promote metabolic dysfunction, and will ultimately lead to the death of the afflicted individual if left untreated[14,15]. Cancer is one of the most prevalent worldwide diseases that resulted in approximately 19.3 million new diagnoses and 10 million deaths in the year 2020 alone[16]. Cancers of the blood cells are broadly termed as “leukemia”, which includes several main types

known as ALL, acute myeloid leukemia (AML), chronic lymphocytic leukemia (CLL), and chronic myelogenous leukemia (CML)[17]. As ALL is the most common leukemia diagnosis in children, it will remain the primary focus of this thesis, though considerations for the other types of leukemia remain warrant for thoughtful consideration[5].

Medical surgeon Peter Cullen gave the first known description of what was most likely a case of chronic leukemia in 1811[18,19]. A 35-year-old male patient who had a notable fever and abdominal pain was treated with the mostly archaic procedure of blood-letting, in which the patient was purposely made to bleed in an effort to "cure" them of their suspected disease[20]. Cullen noticed that his patient's blood presented as a milky white color, likely due to the high presence of white blood cells (WBCs), a trademark feature of leukemia. Shortly thereafter in 1827, another surgeon known as Alfred Velpeau had a 54-year-old male patient who presented with similar symptoms to Cullen's patient, with the additional presence of urinary stones[18]. Upon autopsy, Velpeau noticed that the patient's liver and spleen were abnormally enlarged, and his blood also had a thickened, pus-like appearance. It was immediately clear that these two patients had something wrong with their blood supply. Cullen inaccurately hypothesized that the milky serum was related to a strange absorption of fat, while Velpeau correctly reasoned that there was an increase in WBCs, and that this disease was likely associated with the circulatory system[18,21].

Several other similar cases were observed over the duration of the 19th century, some of which may or may not have been actual cases of leukemia. Nevertheless, a gradual understanding of this peculiar disease was collected with scientist Alfred Donné being possibly the first to detail the leukemic cell morphology, including the presence of large cytoplasmic space that he observed under the microscope in 1844[18]. Donné accurately proposed the idea that

the increase in white blood cells resulted from a problem in which cell differentiation was arrested, which is now known to be the hallmark predisposition for leukemia[18,22].

Despite these notable advancements in the knowledge of leukemia, it took decades for scientists and physicians to really grasp what was happening at the cellular level and to provide somewhat reliable treatments for the various types of leukemia. Several treatment attempts were made over the 19th and early to mid-20th century, including blood-letting, provision of iron supplements, radioactive phosphorus exposure, electromagnetic radiation therapy, and eventually chemotherapy, with the components and dose recommendations changing as more information was gathered[18]. However, to properly target any disease, its pathogenesis must be understood so that observed alterations in metabolism, including downregulation and upregulation of metabolites, misfolded proteins and resulting enzymatic products, and general DNA defects that contribute to the development and maintenance of the disease in question, can be manipulated for therapeutic benefit.

1.2.2 ALL pathogenesis

Leukemia is a complex group of cancers that originate in the bone marrow, in which its four main types of ALL, AML, CLL, and CML are categorized and diagnosed based on the pathway of interrupted cell differentiation, the resulting abnormal blood cell count, WBC morphology differences, and the presenting symptoms in the patient[23]. The etiology of ALL is mostly unknown, with certain conditions such as Down syndrome, exposure to radiation, or even birth through caesarean section proposed to be possible risk factors[24,25]. ALL develops because of a dysregulation within the lymphoid vein of the hematopoiesis pathway, or blood cell differentiation pathway. The lymphoid progenitor, a cell that normally differentiates into immune

B-cells and T-cells, is typically tightly regulated by transcription factors and signal transduction[26,27]. In the case of ALL, this cell maturation process is abruptly halted and becomes largely dysfunctional, resulting in an accumulation of premature B-cells or T-cells that are classified into over 30 different subtypes of ALL based on their protein expression, also known as their immunophenotype[28,29]. See **Figure 2** for a simplified diagram of the normal hematopoietic pathway versus ALL development.

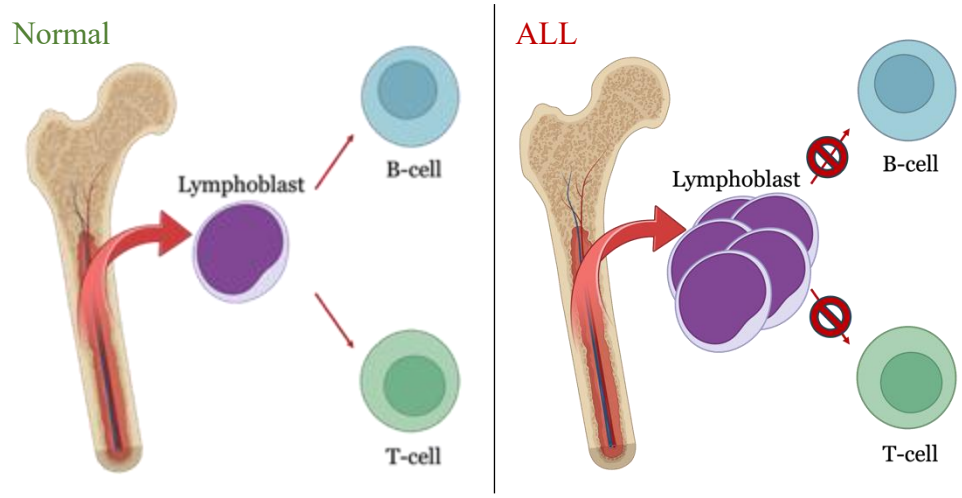

Figure 2: Simplified Normal Hematopoietic Pathway versus ALL Development

A simplified diagram of the normal hematopoietic pathway versus the dysregulated hematopoietic pathway that leads to the development of ALL. Lymphoblasts produced in the bone marrow typically differentiate into immune B-cells and T-cells. When this hematopoietic process is blocked, lymphoblasts fail to differentiate as normal and instead accumulate in the peripheral blood as immature B-cells or T-cells, leading to the cancerous condition known as ALL that is fatal if left untreated.

Several mutations that result in an overproduction or underproduction of chromosomes, known as hyperdiploidy and hypodiploidy respectively, or the rearrangement, translocation, or deletion of certain genes, are known to be present in ALL[30]. Specifically, in B-cell ALL (B-ALL), transcription factors such as paired box protein 5 (PAX5), IKAROS family zinc finger protein 1 (IKZF1), transcription factor 3 (TCF3), and early B-cell factor 1 (EBF1), are responsible for B-cell differentiation and maturation through the activation and repression of relevant genes[31–36]. In the case of T-cell ALL (T-ALL), transcription factors such as GATA-binding protein 3 (Gata3) and RUNT-related transcription factor (Runx) play a similar role, however, T-cell differentiation may be less clearly defined[37,38]. If these key transcription factors lose their expression or become non-functional, the doorway to leukemia development opens.

According to the most recent statistics provided by the Canadian Cancer Society (CCS), 440 Canadian adults, accounting for 245 men and 195 women, were diagnosed with ALL in 2019[39]. This incidence rate is consistent with the literature, which shows that males are slightly more likely to develop ALL than females[40,41]. In the same year, 205 children were diagnosed with ALL out of a total of 250 new childhood leukemia cases across Canada, demonstrating an astonishingly high prevalence of ALL in the country[42]. Typically, pediatric ALL patients initially present with a number of afflicting symptoms such as fever, bone pain, fatigue, and easy bruising[43]. Adults with ALL may suffer from similar symptoms, including weight loss and night sweats, though there are some clinical differences[24,44]. For example, adults are more likely than children to have a high WBC count, develop central nervous system (CNS) complications, and have the Philadelphia chromosome (Ph) mutation, which is a reciprocal translocation of chromosomes 9 and 22 that is most often present in CML but also can appear in ALL and AML[44,45]. In contrast, children are more likely to have B-ALL hyperdiploidy at a rate of 30-40%

of cases between the ages of 1-10 years, compared to a rate of <20% in ages 10-15, <10% in ages 15-24, and <5% in ages 25-44[46].

The differences between adult and pediatric ALL are further differentiated in the spotlight of clinical care approaches. Overall, children with ALL respond well to current chemotherapy interventions, with approximately 90% of children entering remission, and 63-83% of those cases remaining disease-free for at least 5 years, while adult leukemia treatment success has a similar 75-89% remission rate, but has lagged behind dramatically with only a 28-39% chance of remaining disease-free for 3-5 years[47]. This is partly due to the fact that treatments have not been optimized as well for adult patients, who require different therapeutic care, perhaps due to differences in their leukemic origins[47]. Also, ALL is much more rare in adults compared to children, and perhaps takes on a more complex disease pathogenesis.

1.2.3 ALL treatments

Owing to the complexity and variety of each individual leukemia diagnosis with its dozens of subtypes, current treatment protocols are tailored for patients based on their associated clinical features, type of leukemia cells, and initial response to treatment[7]. Eventually, with an increased understanding of pharmacogenomic medical practices in which treatments are personalized according to a patient's genetic biomarkers, chemotherapy regimens will preferably become better at promoting remission, while avoiding ADRs[48]. Presently, treatment protocols for ALL include a variety of drugs such as asparaginase, anthracyclines, methotrexate, thiopurines, vincristine, and glucocorticoids[48]. While all of these drugs carry their own unique set of potential ADRs, asparaginase is of particular interest as the ADR of pancreatitis can result in retraction of

this crucial therapeutic component, which may make children more susceptible to leukemia relapse[49].

For the purposes of this thesis project, the focus is solely on understanding the mechanisms behind the development of the ADR known as asparaginase-induced pancreatitis (AIP) in children. The influence of asparaginase on ALL treatment success in children has been one of the best recorded cancer intervention achievements in history, yet it still has to make substantial progress before it can be declared a full cure with little to no serious side effects. There are multiple formulations of asparaginase available on the Canadian market and all are known to cause pancreatitis, but the mechanisms through which this ADR develops are not fully understood. A recently approved asparaginase variant known as Asparlas® is expected to replace the most common formulation currently used, Oncaspar®, as it has a longer half-life and fewer infusion requirements[50]. However, Asparlas® has been shown to be associated with an increased risk of pancreatitis development, which is cause for concern[51]. To aid the ongoing effort to improve asparaginase safety while maintaining or increasing efficacy, the experimental goal was to investigate the unexplained and dangerous yet considerably common association between asparaginase and pancreatitis.

1.3.0 Asparaginase

1.3.1 Asparaginase history and clinical establishment

One of the therapeutics most responsible for the vast improvements in the treatment of ALL is asparaginase. Fascinatingly, the use of asparaginase as a cancer treatment was discovered in an unconventional manner. In a ground-breaking experiment performed in 1953, Kidd et al. were investigating possible treatments for mice that had induced lymphoma, another type of

blood cancer that originates from mature B-cells and T-cells that become malignant and typically centralize in the lymphatic tissue[52,53]. They intraperitoneally (IP) injected the mice with either guinea pig, horse, or rabbit serum, or saline as a control. Surprisingly, the guinea pig serum was shown to reduce the size of the lymphoma tumors in the mice, while the other mice continued to have progressive tumor growth and eventually died[53]. At the time, the therapeutic effect brought on by the guinea pig serum was not understood at a molecular level, but it was immediately recognized to be a research area of vital importance. Interestingly, Kidd et al. also noted that the mice did not develop ADRs of arthritis or kidney inflammation, or show any other microscopic signs of biological stress when given repeated doses of the guinea pig serum[53].

In 1961, Broome et al. gathered evidence to suggest that activity of the enzyme L-asparaginase, responsible for the conversion of the amino acid L-asparagine into L-aspartic acid and ammonia (NH_3), was the component in guinea pig serum responsible for its anti-lymphoma effects[54,55]. In fact, the presence of high L-asparaginase activity in guinea pig serum had been observed decades before, in 1922, when it was simultaneously reported to be absent in the serum of other mammal species[56,57]. Furthermore, the total circulating concentration of L-asparaginase in guinea pigs increases with age, with neonatal guinea pigs expressing only 3-15% L-asparaginase activity compared to adults[58]. However, it remains unclear as to why guinea pigs specifically have such a substantially high expression of L-asparaginase. There are also other groups of organisms known to produce L-asparaginase, including different species of bacteria, plants, and yeast[59]. As the clinical relevance of L-asparaginase in the treatment of ALL started to become clear, the mass production of guinea pigs for L-asparaginase harvesting was certainly impractical, and researchers turned their focus to bacteria.

Specifically, the bacteria *Escherichia coli* (*E. coli*) were not only able to produce overall larger quantities of L-asparaginase more efficiently but were also deemed to have more favorable L-asparaginase properties compared to that of guinea pigs, such as a lower molecular weight and better solubility when half-saturated in 15% sodium sulfate[60]. Therefore, *E. coli* L-asparaginase was assumed at the time to be a more effective anti-lymphoma agent than guinea pig L-asparaginase[60]. However, a returned interest in favor of guinea pig L-asparaginase suggests that it may be a less immunogenic option than bacteria-derived sources as it shares up to 88.6% structural similarity to a human enzyme known as 60-kDa lysophospholipase[61]. In fact, the first human trial performed in 1966 on an 8-year-old male with ALL was conducted using guinea pig serum as the L-asparaginase source[62,63]. Sadly the child died 10 days post-treatment, however, there were notable clinical signs of improvement, such as a decrease in WBC count, and reduction of tumor growth[62].

A second L-asparaginase trial on three pediatric patients in 1967 also demonstrated clinical improvement in all patients, with one child entering temporary partial or full remission[63,64]. Unfortunately, as L-asparaginase production was scare at the time, supplies ran out during the trials, and two of the patients passed away[64]. Nonetheless, these preliminary studies gradually paved the way to the FDA approval of *E. coli*-derived L-asparaginase in 1978, under the name Elspar®[63,65]. This single decision forever changed the mortality profile of pediatric ALL patients for the better, but presented doctors and researchers with a new set of ADR challenges, which are still largely under investigation today.

1.3.2 Asparaginase mechanism of action and adverse reactions

L-asparaginase as a biologic mechanistically works in a unique way compared to other chemotherapeutic agents, which are typically cytotoxic and often interfere directly with a cancer cell's receptors or DNA. Instead of molecularly interacting with the leukemic cell components, L-asparaginase targets one of its essential nutrients, the amino acid L-asparagine. As asparagine is a non-essential amino acid, normal body cells can generate their own supply of asparagine that they need for metabolic processes through an enzyme known as asparagine synthetase (ASNS), but ALL leukemic cells either completely lack this enzyme or express it in very low quantities[66,67]. Therefore, ALL leukemic cells must uptake exogenous asparagine from the blood stream, which may be sourced from the patient's diet, healthy cells, or gut microbiota[68]. L-asparaginase hydrolyzes L-asparagine into L-aspartic acid and NH_3, which deprives leukemic cells of the asparagine nutrients needed to maintain their rapid proliferation demands, and consequently starves them to death[66,69].

Nonetheless, although L-asparaginase isolated from bacterial sources is quite specific at hydrolyzing L-asparagine, it is also known to have L-glutaminase activity, in which it hydrolyzes L-glutamine into L-glutamic acid and NH_3[66]. This lesser reaction, accounting for about 2-10% of the total activity of bacteria-derived L-asparaginase formulations approved by the FDA, is thought to be a contributing factor to some of the L-asparaginase-related ADRs. For example, L-glutaminase activity potentially plays a role in hepatotoxicity and hyperammonemia, a condition in which accumulating levels of the by-product NH_3 become dangerous and are associated with neurotoxicity[66,70–72]. Furthermore, it has been suggested that L-glutaminase activity is not necessary for the anti-tumor affects of L-asparaginase, and the removal of it or absence of it as

seen in other sources of L-asparaginase is a research point of interest[73,74]. In conflict, however, is the argument that depleting glutamine is necessary for anti-cancer activity[73].

As with most cancer treatment interventions, asparaginase is known to cause ADRs in pediatric patients, which likely cannot all be explained by L-glutaminase activity alone. Some of the most prevalent ADRs include hypersensitivity, pancreatitis development, hyperglycemia, usually when taken in combination with glucocorticoids, thromboembolic events, and different forms of liver dysfunction or hepatotoxicity[12,57,75–83]. See **Table 1** for a detailed comparison of the prevalence of these ADRs as reported by multiple clinical studies and review articles. Drug companies have attempted to combat hypersensitivity specifically by sourcing asparaginase from different bacterial strains. However, immunogenic reactions continue to be problematic, despite these modifications. Pegylated versions of asparaginase that have assisted in increasing the half-life of the enzyme have also reduced the likelihood of developing hypersensitivity in some literature reports, while conflictingly increasing it in others, as polyethylene glycol (PEG) itself is known to be an allergen[84]. At present, although the ADRs induced by asparaginase can be managed, it would be largely beneficial for them to be prevented altogether, as the severity of the ADR can lead to the termination of crucial asparaginase administration.

Table 1: Prevalence of Common Asparaginase-associated Adverse Drug Reactions Across Multiple Studies and Review Articles

Prevalence of common ADRs associated with asparaginase administration as reported across mulitiple clinical studies and reviews. The different incidence rates can vary depending on the population observed, the administration protocol, etc.

		Adverse Drug Reaction and Corresponding Prevalence (%)				
Study	**Publication type**	**Hypersensitivity**	**Pancreatitis**	**Hyperglycemia**	**Thromboembolic events**	**Hepatotoxicity**
Aisyi 2019	Clinical	-	-	5.2%	-	-
Ahmad 2018	Review	-	-	10-15%	-	-
Ben Tanfous 2015	Clinical	15.8%	5.6%	-	3.5%	-
Duarte 2016	Clinical	-	-	-	3.8%	-
Fonseca 2021	Review	30-75%	-	-	-	-
Gibson 2021	Review	-	1.5-18%	-	-	-
Hijiya 2016	Review	3-75%	2-18%	4-20%	2-7%	4-60%
Højfeldt 2019	Clinical	13.8%	-	-	-	-
Raja 2014	Clinical	-	5.9%	-	-	-
Schmidt 2021	Clinical	24.1%	3%	4.2%	3.6%	19.4%

Currently there are three asparaginase formulations that are actively being used for the treatment of pediatric and/or young adult ALL in Canada, age 1 month to 21 years. Oncaspar®, a pegylated version of asparaginase, is the most commonly used formulation. Rylaze™, a recombinant form of L-asparaginase from *Erwinia chrysanthemi* (*Erwinia*) that is instead produced in *Pseudomonas fluorescens*, is used as a second-line treatment when hypersensitivity develops to Oncaspar®[85]. The most recent pegylated formulation approved in Canada as of 2023, Asparlas®, is expected to surpass Oncaspar® as the primary asparaginase drug, with Asparlas® having better drug stability and a longer half-life[86]. Oncaspar® and Asparlas® both contain L-asparaginase linked to a similar monomethoxy PEG component, but Oncaspar® contains a succinimidyl succinate linker, while Asparlas® contains a succinimidyl carbonate linker[87]. The linker seen in Asparlas® is less susceptible to enzymatic hydrolysis and thus makes the compound more stable[87]. Unfortunately however, although they can vary in their ADR profiles from one another in terms of prevalence, all formulations of asparaginase thus far produce similar toxicities, with pancreatitis remaining at the top of the list.

1.4.0 Pancreatitis, an ADR of asparaginase

1.4.1 AIP mechanisms

The current knowledge surrounding the mechanism(s) through which AIP develops and its associated risk factors is limited. Pancreatitis, in general, is a condition in which the pancreas becomes inflamed and can release digestive enzymes in an incorrect fashion, leading to malabsorption of food and subsequent nutritional and digestive issues[88]. Ultimately, pancreatitis can be life-threatening when metabolic complications linger, leading to conditions such as localized necrosis or wide-spread organ failure[88–90]. Overall, up to 5% of pancreatitis cases are

fatal with morbidity increasing to 30% amongst severe cases, and chronic pancreatitis can lead to an increased risk of developing pancreatic cancer[91]. Numerous agents and diseases that are known to cause pancreatitis include excessive alcohol use, gallstone disease, viral infections, medicinal drugs like asparaginase and tetracycline, hypertriglyceridemia, and hypercalcemia[92–94]. In rare cases, pancreatitis can even be hereditary and reoccur frequently[92,95,96].

Scientists have only conducted a handful of cellular and animal studies that have lead to a preliminary theoretical understanding of how asparaginase induces pancreatitis[97–100]. In isolated mouse pancreatitc acinar cells, asparaginase has been shown to induce pancreatitis in a fashion similar to other pancreatitis-inducing agents[97]. Normally, the pancreas will release digestive enzymes through the trigger of short-lasting cytosolic calcium (Ca^{2+}) signalling, which temporarily increases adenosine triphosphate (ATP) production[97]. One hypothesis suggests that AIP develops through the unusual sustained release of Ca^{2+}, which when combined with reactive oxygen species (ROS) and reactive nitrogen species (RNS) results in abnormal mitochondrial Ca^{2+} uptake, and decreases ATP production[91,97]. The lack of ATP hinders extrusion of Ca^{2+} from the cell, and the toxic levels of Ca^{2+} subsequently lead to necrosis of the pancreatic cells[97]. Asparaginase has been shown to elicit this dangerous Ca^{+} response by acting on protease-activated receptor 2 (PAR2), but its effects can be reduced by blocking Ca^{2+} release-activated Ca^{2+} (CRAC) channels[97].

A second hypothesis suggests that AIP develops as a result of the asparagine depletion, leading to a nutrient stress response known as the amino acid response (AAR). It has been shown *in vitro* that normally the AAR will promote the upregulation of pancreatitic ASNS, which in turn can protect the pancreas from injury. If ASNS upregulation is somehow blocked during exposure to asparaginase, this could lead to AIP development and a defective AAR can also

promote hepatotoxicity and immunosuppression[98,100]. It is possible that both hypotheses could occur simultaneously. Regardless, although the pathogenesis of AIP may be at least partially understood, its full mechanisms remain a mystery.

1.4.2 Clinical diagnosis, treatment, and risk factors of AIP

AIP is diagnosed based on the presence of at least two out of three key markers. Patients must either present with 1) symptomatic abdominal pain, 2) physiological changes of the pancreas as imaged by ultrasound, CT scans, or MRI, and/or 3) three-times higher than normal levels of the pancreatic digestive enzymes amylase and lipase[101,102]. These enzymes are secreted via the pancreatitc acinar cells into the pancreatic ducts and intestinal tract through an exocrine mechanism, whereas other enzymes like insulin are secreted via the pancreatitc beta cells directly into the interstitum, and diffuse into the bloodstream via an endocrine pathway[103–105].

Futhermore, clinicians use grading scales to determine the severity of the AIP presentation. One such example of a grading system used, summarized in **Figure 3**, categorizes children into Grade 2 when they are asymptomatic but their diagnosis of pancreatitis necessitates temporary withholding of asparaginase until their pancreas returns to normal. When AIP symptoms of pain are present for <72 hours, children are moved into the Grade 3 mild category, and some planned doses of asparaginase will be omitted, even after recovery. Grade 3 significant pancreatitis is determined when symptoms last for >72 hours or further complications surface, while Grade 4 severe pancreatitis is pronounced when the AIP has reached the life-threatening threshold. In both of the latter cases, asparaginase must be permanently discontinued, accounting for an increased risk of death and ALL relapse.

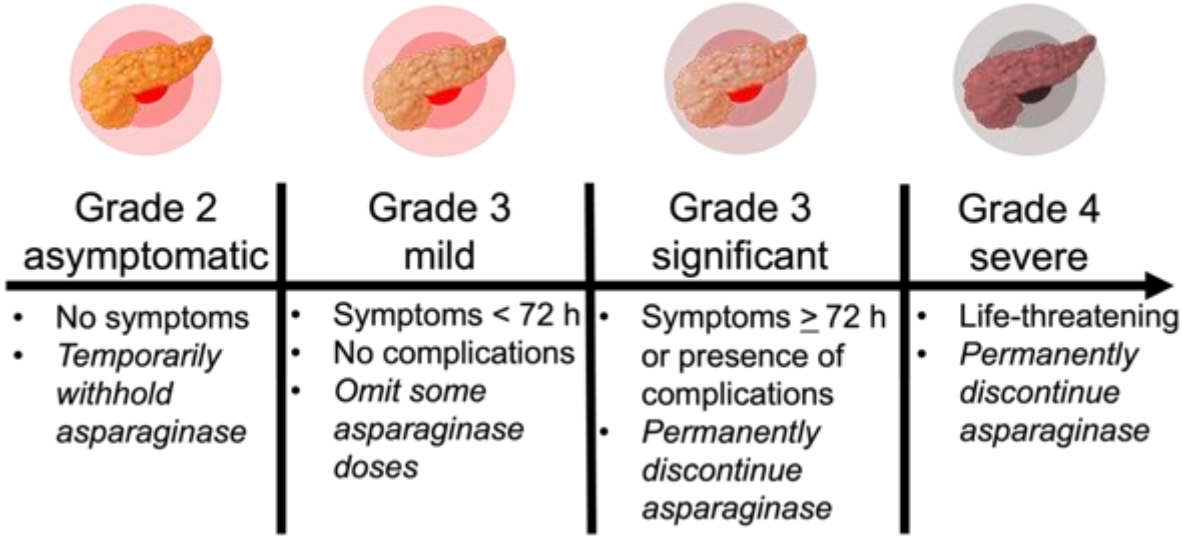

Figure 3: AIP Grading Scale

AIP severity is categorized into 4 main groups that assess the presence of symptomatic pain and complications to determine whether asparaginase treatment should be temporarily withheld, have some doses omitted from the therapeutic plan, or permenantly discontinued altogether to prioritize the physical safety of the patient.

AIP is treated via supportive care, including fluid resuscitation, pain management, and antibiotics[106]. Furthermore, AIP can result in acute complications in which mechanical ventilation or insulin therapy may be needed, and/or pancreatic pseudocysts may form, which are fluid amylase-filled encapsulations that can cause their own set of complications after the clearance of pancreatitis[107,108]. Some patients may have to stay on insulin treatment long-term, and painful abdominal episodes can continue to occur for over a year post-asparaginase treatment[107]. Thus, AIP not only poses a serious healthcare burden on the patient and their family, but can also cause a significant financial burden on the medical system in terms of continued supportive care.

Some risk factors for AIP development have been investigated and proposed, but a widespread consensus has not yet been reached in the literature. Possible risk factors for pancreatitis development include age, especially between 10-18 years old, the type of asparaginase drug formulation used, total asparaginase dose intensity administered in a single month, and Native American ancestry[12,107,109,110]. Age appears to be a consistent factor, as multiple studies have noted a significant association between older pediatrics and the development of AIP. However, pancreatitis appears to develop within the first few doses of asparaginase, suggesting that there may be some sort of biological predisposition to its development rather than being the result of a cumulative drug exposure effect[12]. Previous development of AIP also does not appear to be a predictor of future AIP establishment when patients are re-exposed to asparaginase, suggesting that some patients may have the ability to adapt to the treatment[107].

1.4.3 Possible genetic polymorphisms involved in AIP

With such unpredictable AIP risk factors at play, it is necessary to garner a better understanding of what is happening between asparaginase and cells at a molecular and genetic interaction level. A few studies have attempted to identify gene variants or single nucleotide polymorphisms (SNPs) associated with AIP. In 2016, Liu et al. studied a cohort of 5185 pediatric patients and young adults with ALL and found that those with a rare nonsense variant in carboxypeptidase A2 (*CPA2*), a pancreatic enzyme involved in food digestion, had an increased risk for the development of pancreatitis[111,112]. Another study published in 2017 by Wolthers et al. found that more than half of the AIP cases in a pool of 1285 children carried a genetic variant in either the unc-51-like kinase 2 (*ULK2*) gene involved in autophagy, or the regulator of G-protein

signaling 6 (*RGS6*) gene involved in G-protein signalling, or both[113]. However, the same researchers published more data in 2019, and claimed that they failed to validate their previous results, while identifying 30 novel top SNP hits that were most associated with AIP[114].

Before the start of this thesis project, an ongoing pharmacogenomic study performed by the Canadian Pharmacogenomics Network for Drug Safety (CPNDS) identified possible genetic links between asparaginase and the likelihood of developing subsequent pancreatitis. An initial data analysis on 63 pediatric patients who developed AIP compared to 937 matched controls showed that genetic variants in paired-like homeodomain 2 (*PITX2, OR: 8.6; 95% CI:3.6-20.7*) and retinoic acid receptor beta (*RARβ, OR: 10.9; 95% CI:4.3-27.9*) were strongly associated with pancreatitis development. The transcription factor *PITX2*, which plays an essential role in developing regions of the body such as the abdominal wall, has recently been shown to act as an oncogene in pancreatic cancer[115]. *RARβ*, also a transcription factor, has shown to be involved in embryonic stem cell differentiation into pancreatic islet cells, and vitamin A-dependent immunity[116,117]. Based on these preliminary findings, we hypothesized that *PITX2* and *RARβ* are involved in the development of AIP. Subsequently, their gene expression could be down- or up-regulated in the pancreas when exposed to asparaginase, leading AIP development. We decided to work towards an understanding of this hypothesis by developing an AIP mouse model and measuring the mRNA expression of both *PITX2* and *RARβ*.

1.5.0 Mice as models of research

1.5.1 Mice versus humans

To appreciate the research herein, an understanding of the choice for the use of mice in parallel to humans must be comprehended. It is important to note that although mice are not a

perfect translatable model to humans, they remain one of the most viable research options in modern science, and have paved the way for many discoveries and medical innovations[118].

Even though ALL is relatively common in children with cancer, accounting for approximately 25% of all cases, it remains a proportionally rare disease, as all leukemias only affect approximately 4.9 in 100,000 children and adolescents under 20 years old[5,119]. Therefore, studying the genetic makeup of children with leukemia who specifically develop AIP is understandably a very slow process.. If an animal model such as mice, who have around 95% of the same genetic makeup as humans, can be used to investigate similar abnormalities in vivo, scientists can arrive at an answer much more efficiently[120].

Furthermore, mice have been used numerous times as acute and chronic pancreatitis research models using other pancreatitis-inducing agents such as alcohol metabolites, bile salts, the drug cerulein, and a choline deficiency diet [121,122]. Mice are also an ideal economical model in terms of *in vivo* research, and multiple animals can be conveniently studied in replicates at once to produce statistically significant results. Finally, mice are quite accessible and ideal for preclinical trials and post-marketing drug surveillance research, the latter being the case for asparaginase ADRs.

1.5.2 The current knowledge of AIP in mice

AIP has specifically been explored in mice a mere handful of times, under numerous varying conditions in the literature[98–100,123–128]. The typical dose of asparaginase given to mice is usually 3 IU/g, administered as repeated IP injections, though others have reported daily doses as high as 20 IU/g[98–100,124,127]. Black C57BL/6J (C57) mice have been the most popular potential AIP model of choice, but other strains such as BALB/cByJ (BALB/c), Swiss-Webster, and NRG

mice have also been explored. All mouse studies performed thus far have provided novel insights into either possible treatments for AIP or other asparaginase-associated pancreatic damage, or signalling pathways involved in AIP development, but none have focused on the risk factors relating to susceptibility. The current knowledge of AIP in mice will be presented in this section, along with remaining limitations. See **Table 2** for a comparative summary of the experimental parameters in each study discussed. Key differences to note between these studies is that a variety of dosages and formulations of asparaginase have been used, along with inconsistent dosing intervals, treatment time lengths, and administration routes. Also, strains of mice have been picked for unspecified reasons, with ages of mice differing between research groups.

Table 2: Comparison of Experimental Parameters in Mouse Asparaginase/Pancreatic Studies

The experimental parameters are summarized and compared in studies that have specifically looked at AIP or asparaginase-induced pancreatic injury thus far. A dash (-) indicates that the data was not shared in the publication or was represented in inconvertible units, an asterisk (*) means that the information was available but lacked full clarity, or was assumed based on other information provided within the article. All mice ages were converted to weeks, and asparaginase doses were converted to IU/g.

Study	Strain	Age (weeks)	Sex	Dose (IU/g)	Total # of doses	Injection	Formulation	Final timepoint since first dose (days)	AIP?
Kaya 2015	BALB/c	12-14	M	10	1	IP	*E. coli*	5	Unclear
Kose 2016	BALB/c	8	M/F	3	6	IP	-	14	Unclear
Phillipson-Weiner 2016	C57 (*ΔGcn2*)	8	M/F	3	8	IP	*E. coli*	8	Likely
Peng 2018	C57	-	-	20	4	IP	-	4	Yes
Mukherjee 2020	Swiss-Webster	*16-20	F	3	5	-	-	5	*No
Halbrook 2022	C57	*8	*F	-	*5	IP	Pegylated	*15	No
Bollino 2022	NRG	-	-	0.2	4	IV	*Erwinia*	*33	No
Tsai 2023	C57	>8	-	3	8	IP	*E. coli*	8	*No
He 2024	C57	4-6	M	1	-	IM	Pegylated	-	*Yes

The first study that specifically investigated the effects of asparaginase on pancreatic injury in a mouse model dates back to 2015, though earlier research was performed on rats[123,129]. The primary goal of researchers Kaya et al. was to see if they could prevent the development of acute pancreatic injury in mice, rather than focusing on optimizing an AIP model, understanding how AIP develops, or providing post-disease treatment. Male BALB/c mice aged 12-14 weeks were selected as the model of choice, and were divided into four groups; group I received an IP injection of 0.02 mL/g, 0.9 % NaCl saline as a control, group II received daily injections of 500 mg/kg L-carnitine for 5 days, group III received a single injection of 10,000 IU/kg (10 IU/g) *E. coli*-derived asparaginase, and group IV received daily injections of L-carnitine for 5 days before a final single injection of asparaginase[123].

In summary, L-carnitine, an essential participant in fatty acid metabolism that is synthesized endogenously in humans and obtained from the diet, was shown to have a protective affect against asparaginase-induced pancreatic injury when mice in group IV were pretreated with it before receiving a single dose of asparaginase[123,130,131]. Their pancreases had a similar appearance to control group I and L-carnitine group II alone, suggesting that L-carnitine was able to preserve pancreatic cells from asparaginase-induced damage. However, it is notable to point out that although the group III mice treated with asparaginase alone exhibited histological signs of pancreatic damage, including vacuole formation in acinar cells and diffuse necrosis, it is unclear if they developed pancreatitis specifically[123]. When amylase levels were tested, group III mice actually had a significant decrease in amylase levels, rather than an expected increase, but a decrease can indicate the presence of chronic pancreatitis[132]. Acute pancreatitis; however, is the type that initially presents itself in AIP and is cause for concern as it can eventually lead to

further complications like chronic pancreatitis[133]. Thus, the initial response of acute pancreatitis as seen in human clinical settings was not successfully observed directly in this study.

In conclusion, Kaya et al. demonstrated a possible preventative measure with the use of L-carnitine that could eventually be translated to the clinic. However, several unknowns remain. Only a single dose of asparaginase was given to the mice, and thus it is unclear if they would have to be continually simultaneously administered L-carnitine during subsequent doses of asparaginase to prevent pancreatic toxicity, or if a change in dose would make L-carnitine protection unproductive. This research also does not address the issue of how to respond to pancreatic damage or AIP once it has developed, and L-carnitine should have been explored as a post-AIP treatment to see if pancreatic damage could be reversed. None of the mechanisms regarding asparaginase-induced pancreatic injury were investigated either, which makes this study unable to provide any insight into identifying potential pancreatitis risk factors in patients.

Subsequently, research performed by Kose et al. looked at the effects of asparaginase, the corticosteroid prednisolone, and the antilipidemic gemfibrozil separately and in combination to monitor the effects on pancreas and liver histology and lipids. Using male and female BALB/c approximately 8 weeks old, an unidentified formulation of asparaginase was administered 3 times a week for two weeks at a dose of 3 IU/g each time, prednisolone as a single dose of 5 mg/kg, and gemfibrozil as a single dose of 100 mg/kg[124]. In the interest of asparaginase treatment alone and in combination with the other drugs with a focus on the pancreas and liver, asparaginase was suggested to be the cause of significant balloon degeneration, a type of cellular swelling seen in steatohepatitis in the liver[134]. Pancreatitis, on the other hand, was said to be rarely observed in the experiment, and it is unclear if it was present at all in the treatment group that received asparaginase alone.

In contrast, Phillipson-Weiner et al. were the first to clearly suggest that they had observed at least an early form of AIP in a mouse model. However, it is necessary to point out that this observation was only made in their general control nonderepressible 2 (GCN2)-deleted (*ΔGcn2*) C57 mice, and not in the wildtype strain (**Table 2**). GCN2 is a protein kinase that is essential for allowing healthy cells to survive the stressful AAR that is induced through asparaginase depletion of amino acids in the body during treatment[98]. Male and female wildtype and knockout mice were treated with 8 daily injections of either PBS alone or 3.0 IU/g *E. coli*-derived asparaginase, and were dissected 8 hours after the last injection. The pancreases of the *ΔGcn2* mice treated with asparaginase were found to weigh significantly more than their wildtype counterparts, which was one indication that the mice had indeed developed pancreatitis[98]. Amylase levels, notably, were not increased in any asparaginase-treated mice, but histology revealed weak hematoxylin and eosin (H&E) staining of zymogen granules, a pancreatic organelle that releases digestive enzymes, which is characteristic of pancreatitis[98,135]. There were also several cytoplasmic vacuoles in these pancreases as observed via electron microscopy, indicating that the cells were undergoing autophagic cell death, a common feature associated with pancreatitis[98]. In summary, Phillipson-Weiner et al. demonstrated that GCN2 likely plays an essential role in protecting the body from developing AIP. Thus, mutations in any components related to GCN2 function may pose an increased risk of pancreatitis development.

Peng et al. took a different approach, when they intelligently decided to create an AIP mouse model based on the techniques used for established pancreatitis models induced via alcohol metabolites, bile, or cerulein, an agent that increases digestive enzyme secretion[99,136]. Using wildtype C57 mice injected daily for 4 days with the unprecedented high dose of 20 IU/g asparaginase, they were confidently able to capture histological changes in the pancreas

consistent with pancreatitis, including high degrees of necrosis, inflammation, and edema. Peng et al. added to their study by exploring the unique idea of co-treating the mice with galactose, either through adding 100 mM galactose to their drinking water alone, or through drinking water plus a 180 mg/kg/d galactose IP injection. The rational for the use of galactose stemmed from an observation in pancreatic cells that suggested the ATP loss as seen in pancreatitis impaired glucose uptake, prompting the researchers to explore other avenues of regenerating the glycolysis cycle. The control mice, however, only received PBS, and thus there is a pronounced gap in this study as no control mice were treated with galactose. Nevertheless, galactose appeared to provide protective effects against the development of AIP, as galactose-treated mice showed improved pancreas histology, and lost significantly less body weight than mice treated with asparaginase alone[99].

Published research in the area of AIP mouse modelling or associations between asparaginase and effects on the pancreas has more than doubled since the beginning of the 2020s. Mukherjee et al. did not investigate AIP directly but suggested that asparaginase treatment in Swiss-Webster mice significantly increases ASNS levels in the mouse pancreas[100]. This would make sense as the cells are likely trying to compensate for the lower supply of circulating asparagine. Halbrook et al. set out to investigate metabolic programming in pancreatic cancer, but their pegylated-asparaginase (peg-asp)-treated C57 mice did not develop pancreatitis as determined through histological imaging[125]. Bollino et al. comprehensively examined amino acid changes in mice plasma with *Erwinia*-derived asparaginase treatment, but did not address pancreatitis development [126]. Tsai et al. found an association between dietary vitamin A intake and pancreatitis development in humans, with lower dietary vitamin A intake corresponding to the development of AIP[127]. In C57 mice, they found that *E. coli*-derived asparaginase treatment

significantly reduced circulating levels of serum and liver retinol, the main circulating form of vitamin A, which they concluded agrees with the suggestion that retinol is a key component in providing protection against AIP[127,137]. However, they also did not claim that their mice developed pancreatitis. Finally, the latest publication by He et al. appears to demonstrate that C57 mice, who were uniquely administered peg-asp via the intramuscular (IM) route, developed pancreatitis. Histological images of the pancreas were scored and showed that asparaginase-treated animals developed a significant amount of edema and inflammation and had an increase in inflammatory interleukins (IL) 6 and 1β[128,138,139]. Amylase levels, interestingly, did not increase, but lipase levels did, and lipase has been argued to be a more stable indicator of pancreatitis than amylase[90].

In conclusion, only three studies thus far have provided substantive evidence to indicate that their mice developed possible or clear AIP or a possible chronic stage of pancreatitis when treated with asparaginase (**Table 2**). However, Phillipson-Weiner et al. were only able to demonstrate a possible AIP profile in *ΔGcn2* mice and not wildtype mice, and Peng et al. used a high dose of 20 IU/g that could correspond to supra physiologic dosing in humans and may not be clinically relevant. In contrast, the most recent study performed by He et al. that was published during the writing of this thesis may have provided a great amount of insight into the development of an AIP mouse model. He et al. uniquely administered asparaginase via the IM route, which corresponds directly to a common administration route used in human clinics, and IM would conveniently avoid the first pass metabolism that IP is subject to. However, their observations were only recorded in male mice, and it would be important to replicate this in female mice, as both male and female patients are affected by this condition and there could be different mechanisms of action at play based on sex. Therefore, there is still a need to develop a

reliable, wildtype, consistent AIP mouse model to be able to properly investigate the development and risk factors associated with AIP. Risk factors are particularly important to explore, as preventative measures are the ideal implementation over post-disease costly and precarious treatments.

1.6.0 Project aims

1.6.1 Research goals and hypothesis

Herein, the current use of asparaginase as it relates to pancreatitis in particular will be discussed, as AIP can dangerously interfere with a child's ability to receive the best possible treatment[12]. As discussed previously, pancreatitis is a relatively common ADR, which effects up to 18% of pediatric patients and interferes with essential asparaginase administration, yet its cause remains largely unknown[106]. Therefore, it is imperative to determine if such potential inherent risks exist and can be prevented, whether they be present endogenously in the genome as in the case of possible *PITX2* and *RARβ* variants, or are influenced by external factors such as diet and exercise, or a combination of all of the above[140,141].

For this thesis project, it was hypothesized that *PITX2* and *RARβ* protect against the development of AIP. To investigate the validity of this theory, the main following aims were explored:

1) Establish a mouse model with AIP.
2) Determine how *PITX2* and *RARβ* mRNA levels change in the AIP mouse model.
3) Explore differences in male versus female mice response to peg-asp.

CHAPTER II: MATERIALS AND METHODS

2.1.0 Animal ethics approval

Animal experiments performed were reviewed and approved by the Dalhousie University Committee on Laboratory Animals (UCLA), and conducted in compliance with the regulations set by the Canadian Council for Animal Care (UCLA protocol #s 22-081 and 23-033).

2.2.0 Chemical and biological materials

Oncaspar® 750 IU/mL was purchased from Servier Canada Inc. (Laval, QC). BALB/c, A/J, and C57 mice were purchased from The Jackson Laboratory (Maine, USA). Phosphate buffered saline (PBS) was purchased from ThermoFisher Scientific (Waltham, MA) or Fisher Scientific (Pittsburgh, PA). RNeasy Mini Kits were purchased from Qiagen (Venlo, Netherlands). Random Hexamer Primer, dNTP Mix, SuperScript™ II Reverse Transcriptase, and RNaseOUT™ Recombinant Ribonuclease Inhibitor were purchased from ThermoFisher Scientific. Nuclease-free water was purchased from Invitrogen (Waltham, MA). SYBR was purchased from Bio-Rad (Hercules, CA). Murine forward and reverse primers were designed via Primer-BLAST, Primer 3, and Ensembl, and purchased from Sigma-Aldrich (St. Louis, MO). All other reagents and supplies were the highest grade available and purchased from commercial suppliers.

2.3.0 Mice experimental groups and environmental conditions

The first set of experiments (Mice 1.0, see **Figure 4**) were conducted to assess and compare AIP in different mouse strains to pick an optimal strain for AIP development and

subsequent further investigations. Male BALB/c, A/J, and C57 mice aged 6-8 weeks were acclimatized to their new environment for at least one full week before handling for experimentation. As C57 mice have been arguably the most studied in the literature as pancreatitis models with or without the use of different asparaginase formulations, they were selected as reference strain[98,99,142–144]. BALB/c and A/J mice were chosen as comparative strains based on the fact that they presented with a greater increase in serum amylase and lipase levels compared to C57 mice in a pancreatitis experiment that used taurocholate as a bile salt irritant[144,145].

All mice strains were housed in standard ventilated cages containing a minimum of 2 and a maximum of 5 mice per cage, and were kept on a 12-hour light/dark cycle at a controlled room temperature of 21°C. All mice were fed a standardized chow diet (5P04 - Prolab® RMH 3500 Autoclavable, LabDiet®), and had ad libitum access to both food and water. Chow was always provided in pellet formation but was supplemented as mash in some cases. Additionally, a diet supplement gel (DietGel® Recovery, ClearH$_2$O®) was available ad libitum starting on Day 3 for the A/J mice, and for the duration of the whole experiment for the C57 mice. This extra food source was added as an animal welfare refinement based on the unexpected dramatic weight loss seen in the peg-asp treated A/J mice.

The second set of experiments (Mice 2.0, see **Figure 4**) were conducted on the A/J strain once they were identified to be a possible AIP model of interest since they had the most severe reaction to peg-asp. Lower peg-asp doses were used to provide better drug tolerability to the animals, and sex differences were explored. Male and female A/J mice aged 6-8 weeks were handled in the same way as the mice in the first set of experiments without the addition of the

diet supplement gel. Two of the male mice were additionally given a single subcutaneous (SC) injection of meloxicam (5 mg/kg) on Day 1 of the experiment to help ease presumed pain.

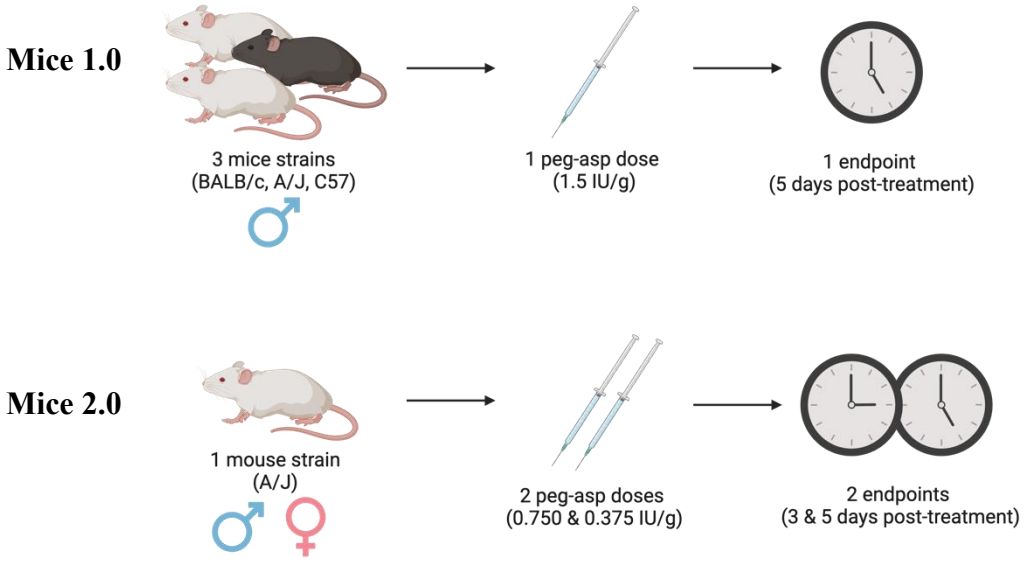

Figure 4: Summary of Mice 1.0 and 2.0 Experiments

Two separate sets of experiments were performed to complete this study. In the first set of experiments (Mice 1.0), 3 strains of male mice including BALB/c, A/J, and C57, were given a single IP injection of PBS or 1.5 IU/g peg-asp and were analyzed at a single endpoint of 5 days post-treatment. This was not part of the plan but a result, no need for it in the methods (agree). In the second set of experiments (Mice 2.0), A/J mice were chosen as the strain to investigate further based on the fact that they presented with the most adverse response to peg-asp. could give the n= (agree) Males and females were given a single IP injection of PBS, 0.750 IU/g, or 0.375 IU/g, and were analyzed 3 or 5 days post-treatment.

2.4.0 Mice experiments, data collection and analyzation

2.4.1 Peg-asp IP dosing

Oncaspar® 750 IU/mL peg-asp was used to create stock solutions dissolved in sterile 1X PBS suitable for 1.5, 0.750, and 0.375 IU/g IP injections into mice. The maximum dose was chosen based on a study that had recently used 1.5 IU/g peg-asp to induce hepatic steatosis, a condition that may have an influence on pancreatitis severity, in C57 mice[146,147]. On Day 0, each mouse was weighed and the volume of stock peg-asp was adjusted for each mouse to receive the approximate injection dose of 1.5, 0.750, or 0.375 IU/g. The corresponding volume of PBS for the control mice was based on the highest peg-asp dose used (1.5 or 0.750 IU/g, depending on the experiment), so that roughly equivalent IP injection volumes were used for most mice. A 25 mm gauge needle was used to administer the peg-asp, and alert mice were typically injected in their lower left quadrant.

2.4.2 Daily weight monitoring and symptomatic observation

Each mouse was weighed daily over the duration of the experiment. A weight loss of ≥15% from the initial body mass was a marker of early humane endpoint (HEP), in which the mouse would have to be euthanized for ethical considerations by the next day if it did not reverse the loss. Changes in physical symptoms and social interactions were also considered, with a score of 0 representing the normal baseline, a score of 1 indicating a possible need to monitor the mouse more frequently or provide supplemental care, and a score of 2 indicating that the mouse had reached a possible HEP (**Table 3**).

Table 3: Monitoring Parameters for Mice Welfare

The following scoring system was used to monitor control and peg-asp-treated mice to determine if they had reached a HEP. Score of 0 for all monitored parameters = proceed with normal once daily monitoring; Score of 1 for one or more monitored parameters = potentially increase monitoring to twice daily and/or provide supplementary care (heat, saline, mash); Score of 2 for one or more monitored parameters (other than weight loss) = consult with veterinary staff and consider HEP; Score of 2 for body weight loss = perform humane euthanasia.

Score	**0**	**1**	**2**
Parameter			
Weight loss	< 10%	10-14%	More than 15 %
Appearance			
Coat	Normal	Mild ruffled coat	Moderate ruffled coat, ungroomed
Body condition	Normal	Thin	Loss of body fat, failure to grow
Body posture	Normal	Hunched	Hunched and still
Movement	Normal	Reduced/slow	Reluctant to move when touched
Activity			
Proximity to others	Close contact	Somewhat separate	Completely separate
Other			
IP Injection site	Normal	Some redness at margins	Redness and swelling

2.4.3 Terminal cardiac puncture, organ dissection and preparation

On Days 3, 4, or 5, mice from each dosing group were anesthetized with 3-4% isoflurane and euthanized using a terminal cardiac puncture, conducted with a 25 mm gauge needle, followed by cervical dislocation. Blood samples were temporarily placed on ice, and centrifuged at 2000 x g for 10 minutes, and the serum collected into separate tubes before storing at -80°C.

Tissue sections from the pancreas and liver were collected, washed in 1X PBS, and immediately fixed in 10% acetate buffered formalin (37% formaldehyde, distilled water, and Na acetate-3H_2O) for between 48-72 hours, followed by 3 washes and finally storage in 70% ethanol for preparation of H&E staining for histology. Additionally, some pieces of the pancreas and liver were snap-frozen in liquid nitrogen before storage at -80°C for mRNA expression analysis.

2.4.4 Histological H&E procedure

H&E histology was performed by the Dalhousie Histology Core. Fixed specimens were processed using the Leica ASP 300 tissue processor. Samples were dehydrated in 70% ethanol for two intervals of 1.5 hours followed by 95% ethanol for two intervals of 1.5 hours, and 100% ethanol for three intervals of 1.5 hours. The samples were then cleared using a 50:50 solution of 100% ethanol:xylene for 1 hour, followed by xylene alone for two intervals of 1 hour. The infiltration or embedding media was created using paraffin wax (tissue prep, Fisher Scientific, melting point 56-57°C) for two intervals of 1 hour. The specimens were then embedded in the paraffin using embedding rings and were orientated to an area of interest. The blocks were solidified after placement at 4°C for 15 minutes. A Reichert-Jung rotary microtome was used to cut 5 μm sections, which were then placed in a 45°C water bath and placed on (+) charged slides.

Slides were dried overnight in a 37°C oven before H&E staining either manually or using the Spectra ST + CV (Leica Biosystems).

2.4.5 Histological microscopy and analyses

H&E slides were imaged using the Zeiss Axio Imager Z1 and Zeiss Axio Observer microscopes with colour cameras, and processed using the Zen 3.0 software. Each image was adjusted for white balance and captured at 20X magnification. Images were blindly analyzed for pancreatitis by Drs. Sohail Husain and Olivia Tsai at Stanford University in California, USA, and reanalyzed by the author. Signs of hepatotoxicity were assessed in our own lab. Pancreases and livers were analysed for features of inflammation/edema or heptatic steatosis, respectively, in comparison to published histological images and other descriptive evidence in the literature[148–160].

2.4.6 Blood enzyme assays

Serum samples were aliquoted and analyzed for total amylase and lipase enzyme activity using the Amylase Activity Assay Kit and Lipase Activity Assay Kit as per the manufacturer's instructions, with slight alterations (Sigma-Aldrich). In the amylase assay, a substrate of amylase , ethylidene-pNP-G7, is cleaved and converted into *p*-nitrophenol. The rate at which ethylidene-pNP-G7 is cleaved by amylase to create 1.0 μmole of *p*-nitrophenol per minute at 25°C is considered to be one unit. The *p*-nitrophenol generated results in a colorimetric product that can be measured at 405 nm. Similarly in the lipase assay, triglycerides which are substrates of lipase are converted into glycerol. The rate at which triglycerides are converted by lipase into 1.0

μmole of glycerol per minute at 37°C is considered to be one unit. The glycerol generated results in a colorimetric product that can be measured at 570 nm.

When multiple samples were run on the same day, a single standard curve in duplicate for each assay was created. The amylase standard curve ranged from 0 – 20 nmole/well of nitrophenol, while the lipase standard curve range from 0 – 10 nmole/well of glycerol. Each individual run thereafter was monitored using a single standard concentration in duplicate to confirm that the samples were still within the linear range. Serum from each mouse sample used was added to the assay at an undiluted volume of 6 μL in duplicate. Absorbance readings at 405 nm for the amylase assay and 570 nm for the lipase assay were obtained using a BioTek Synergy HT plate reader and Gen5 v2.01 software (Agilent Technologies, California, USA). Assays were kept in the plate reader on a kinetic read every 1-5 minutes until the most active sample passed the highest standard's optical density as per the manufacturer's instructions.

Calculations for both amylase and lipase activity followed a similar process. The background was corrected by subtracting the final absorbance reading of the 0 nmole/well standard from the final and initial readings of the standards and samples. The change in final absorbance from the initial absorbance was calculated for each standard and sample and the corresponding optical density was plotted on a linear regression line to interpolate the unknown concentration values of either *p*-nitrophenol or glycerol in the samples. To determine the amylase or lipase activity, the following calculation was used:

$$\text{amylase or lipase activity (nmole/min/mL)} =$$

$$\frac{\text{amount of nitrophenol or glycerol (nmole)} * \text{sample dilution factor}}{\text{reaction time (min)} * \text{ sample volume (mL)}}$$

2.4.7 RT/qPCR analysis

Total RNA was extracted from mice pancreases specimens frozen at -80°C using the RNeasy Mini Kit, as per the manufacturer's instructions. The maximum amount of 30 mg of tissue was used in most cases, though some samples did exceed this. Organs were homogenized for approximately 10 – 30 seconds at 10,000 – 15,000 rpm using a 7 mm stainless steel homogenizer probe (Fisher Scientific). RNA was quantified in a UV transparent microplate (Corning Inc., New York, USA) using a BioTek Synergy HT plate reader and Gen5 v2.01 software. The RNA (~0.5 μg per sample) was reverse transcribed into cDNA using the TProfessional Basic 96 Thermocycler (Montreal Biotech Inc., Quebec, Canada). The cDNA (~1.0 μg per sample) was amplified through qPCR on the Step One Plus real-time PCR thermocycler (Applied Biosystems) using StepOne Software v2.1 in duplicate for each primer set. The amplification protocol included a holding stage at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Each pancreas sample was analyzed for the expression of murine *ASNS*, *PITX2*, *RARα*, and *RARβ*, using *cyclophilin*, *GAPDH*, and *RNA polymerase II* (*RNA poly II*) as the housekeeping genes (**Table 4**). Gene expression was normalized individually to each of the three housekeeping genes, and analyzed via the normalized $2^{-\Delta\Delta CT}$ method[161]. The resulting normalized $2^{-\Delta\Delta CT}$ values for each housekeeping gene were then averaged together and analyzed statistically in GraphPad Prism (version 10.3.0).

Table 4: qPCR Primers Used for Gene Expression Analysis

List of qPCR primers used to determine expression of suspected genes of interest in relation to AIP in mice pancreas samples.

Gene (*Mus musculus*)	PCR Forward Primer (5'-3')	PCR Reverse Primer (5'-3')
ASNS	CACTCCACCACTCCCTTCTT	CCATTTCCACAGACGCAACT
Cyclophilin	GCCATTGCCAAGGAGTAGAG	GTGACTGGCTACCTTCGTCT
GAPDH	TAGTAGCCCAGTGTCCTTGC	TGAACAAGGCCAGAGGAGG
PITX2	TCACCCTTCTGTCACCATCC	GCACCTCCAGTCTATGTTTGG
RARα	CCATCTGCCTCATCTGTGGA	CGGACGTAGACTTTCAGTGC
RARβ	ACCTTGTGTTCACCTTTGCC	TGGCGGTCTCCACAGATTAA
RNA polymerase II	AGAGATGTCTTCCTGGAGCG	CTGTAGCTCTCCTTCCCTGG

2.4.8 Statistical data analysis

Data analysis and statistical calculations were performed using Microsoft Excel and GraphPad Prism. Mice weights between treatment groups were statistically analyzed using a two-way ANOVA and Šídák's multiple comparisons test, and when sex was added as a variable an uncorrected Fisher's LSD was used. Serum amylase and lipase activity levels were analyzed using a two-way ANOVA and Tukey's multiple comparisons test. Liver weights were analyzed via an unpaired two-tailed t-test. The normalized $2^{-\Delta\Delta CT}$ values for qPCR were analyzed using a Kruskal-Wallis test and a one-way ANOVA using the Brown-Forsythe test and Barlett's test.

CHAPTER III: RESULTS

3.1.0: Developing an AIP mouse model

3.1.1 Evaluating the effects of peg-asp-induced weight loss across different mouse strains

Weight loss or gain in animal models treated with an experimental drug can indicate that the drug is having a physiological effect on the animals, either through changing their appetite or altering their metabolism. Asparaginase in particular has been shown to induce weight loss in previous mice experiments[98,99]. Thus, we weighed each strain of mice daily to see if asparaginase was affecting them adversely. Their initial body weight before PBS or peg-asp injection was used as the reference point of 100% body mass. Male BALB/c mice maintained the same weight in both the peg-asp-treated and control groups over 5 days (**Figure 5A**). In contrast, A/J and C57 mice treated with 1.5 IU/g peg-asp lost significant weight compared to their control counterparts after 1- and 2-days post-injection, respectively, and continued to lose weight throughout the duration of the experiment (**Figures 5B,C**). Upon termination of the trial, peg-asp-treated A/J mice had lost 14.8% +/- 1.4 of their original mass over 4 days and had to be euthanized humanely on day 4 as 3/5 mice had reached the HEP. C57 mice lost less weight at 6.1% +/- 1.5 over 5 days. There was no unpredicted mortality.

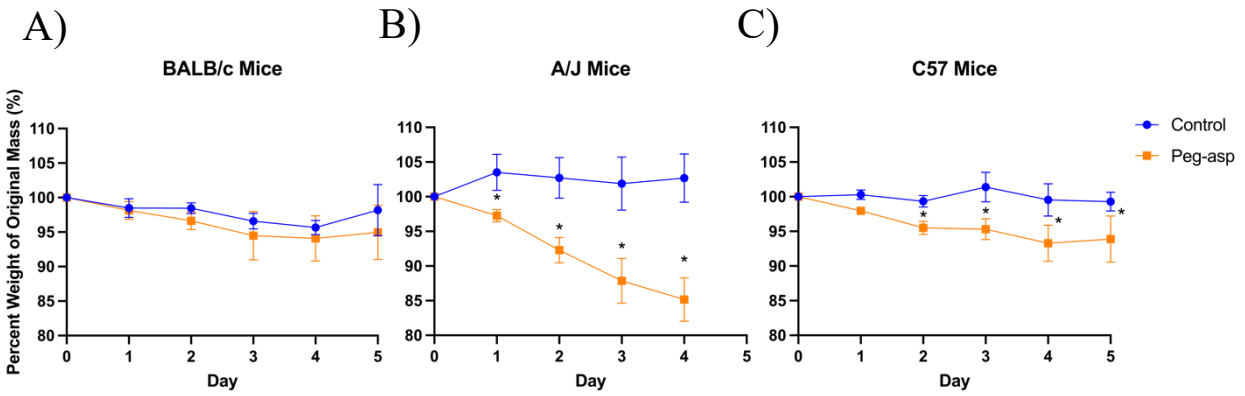

Figure 5: Weight Changes in Control VS Peg-asp-treated Mice over Time

Male BALB/c, A/J, and C57 mice were IP-injected with PBS or 1.5 IU/g peg-asp on Day 0, and weighed daily for 4-5 days until they were euthanized. (A) BALB/c mice exhibited no significant change in weight, while (B) A/J mice and (C) C57 mice both lost significant weight over the duration of the experiment, concluding in the loss of an average of 14.8% +/- 1.4 weight over 4 days, and 6.1% +/- 1.5 over 5 days, respectively. Asterisks (*) indicate a $P \leq 0.05$ as measured by a two-way ANOVA using the Šídák's multiple comparisons test.

When this experiment was repeated for male and female A/J mice treated with 0.750 or 0.375 IU/g peg-asp and dissected on Day 5 (**Figure 6A**) or Day 3 (**Figures 6B,C**), mice treated with 0.375 IU/g peg-asp demonstrated no significant change in weight compared to controls, with the exception of 3-day males, as indicated by the red asterisks. In contrast, mice treated with 0.750 IU/g continued to have a significant difference in total weight loss compared to controls throughout the duration of the experiment, as was observed at the prior higher dose of 1.5 IU/g peg-asp. However, A/J mice did not lose weight as dramatically as previously shown in **Figure 5B**, and some regained weight before the end of the experiment, averaging out to a plateau in **Figure 6A**. Interestingly, female mice gained more weight than their control male counterparts,

and likewise lost significantly less weight than males at both doses of peg-asp at 2- and 3-days post-injection (**Figure 6D**).

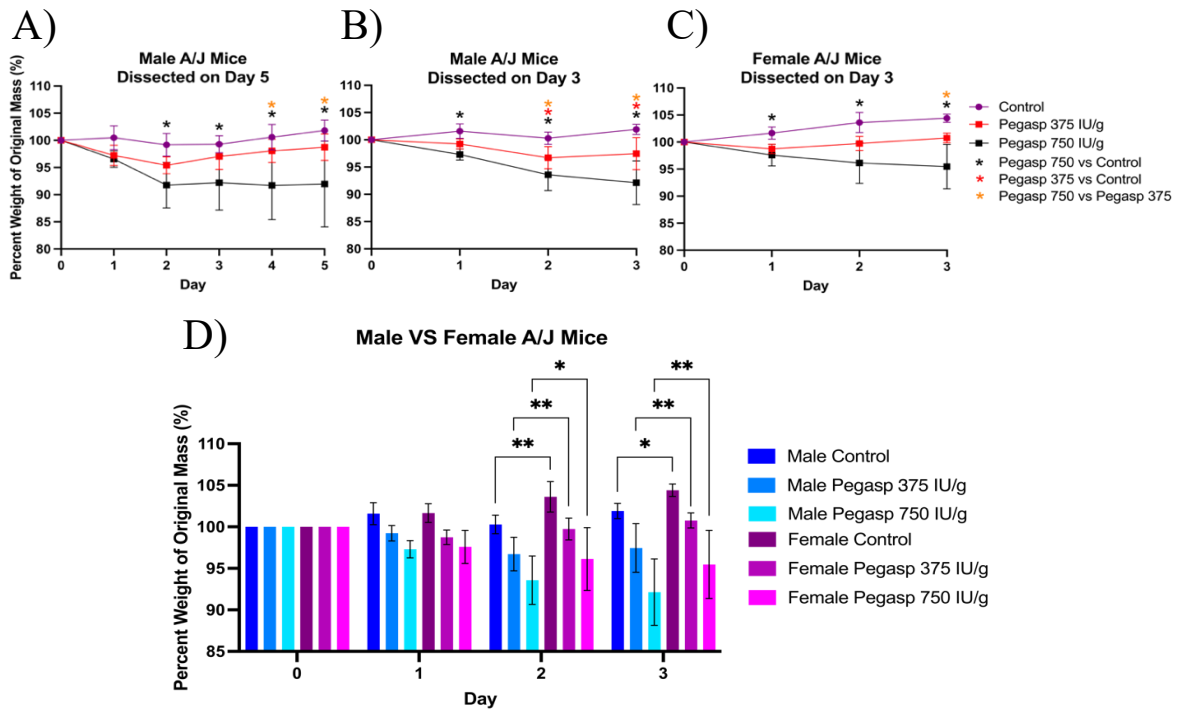

Figure 6: Peg-asp A/J Mice Lose Significant Weight, but Males Lose More than Females

Male and female A/J mice were IP-injected with PBS or 0.750 or 0.375 IU/g peg-asp on Day 0, and weighed daily for 3-5 days until they were euthanized. (A) Male A/J mice had significant weight loss with 0.750 IU/g peg-asp treatment compared to controls (A, B). Male mice showed some significant weight loss 2- and 3-days post-treatment with 0.375 IU/g peg-asp (B). (C) Female A/J mice dissected on Day 3 also lost significant weight at 0.750 IU/g peg-asp compared to controls, but overall, (D) females lost less weight than their male counterparts at all doses 2- and 3-days post-peg-asp treatment. Asterisks (*) indicate a $P \leq 0.05$ as measured by a two-way ANOVA using the Šídák's multiple comparisons test or uncorrected Fisher's LSD.

3.1.2 Investigating diagnostic features of AIP across various mouse strains

A section of each control and peg-asp-treated pancreas from all three mice strains was photographed and blind-labelled before submission to our collaborators Dr. Husain and Dr. Tsai at Standford University for analysis. Secondary analysis was performed by the author who referenced previous pancreatic injury or pancreatitis studies and corresponding H&E histology images to look for key features that could indicate the development of AIP[150,151]. It was concluded by all observers that there was no obvious injury present in the images.. However, some samples appeared to have possibly experienced an AIP episode or shift in pancreatic homeostasis but were demonstrating recovery by the time they were dissected, similar to a pancreatitis model that used cerulein[150]. For example, **Figure 7A** shows a control BALB/c mouse pancreas in comparison to peg-asp-treated BALB/c mice in **Figures 7B** and **C**, which demonstrate features of pancreatitc fat necrosis, and islet cell hyperplasia or enlargening, respectively. Pancreatitis can proprogate fat necrosis, a condition in which surrounding adipocytes are broken down inappropriately by pancreatic enzymes and appear as white chalky deposits in histology, while islet cell hyperplasia can indicate that insulin production has been disturbed in an organism[152–154].

It is important to point out, however, that even some control mice, as seen in **Figure 7A**, potentially exhibited signs of mild edema or inflammation, and **Figure 7D** showing a control A/J pancreas appears to have some pancreas cell necrosis. Nontheless, partial edema or inflammation is more apparent in the displayed peg-asp-treated A/J mice (**Figure 7E**), and another appeared to have substaintial leukocyte infiltration (**Figure 7F**)[155,156]. As for C57 mice, controls versus peg-asp-treated appeared to have very similar phenotypic profiles, with 3/5 peg-asp-treated mice exhibiting weak staining of zymogen granules, determined by Dr. Tsai (**Figure 7G-I**). However,

some control mice pancreases from all strains also presented with similar weak zymogen granules. Thus, based on both the initial and secondary H&E analyses, it cannot be concluded that any mice reached a definitive AIP endpoint and it is possible that some control mice were experiencing unexpected pancreatic stress.

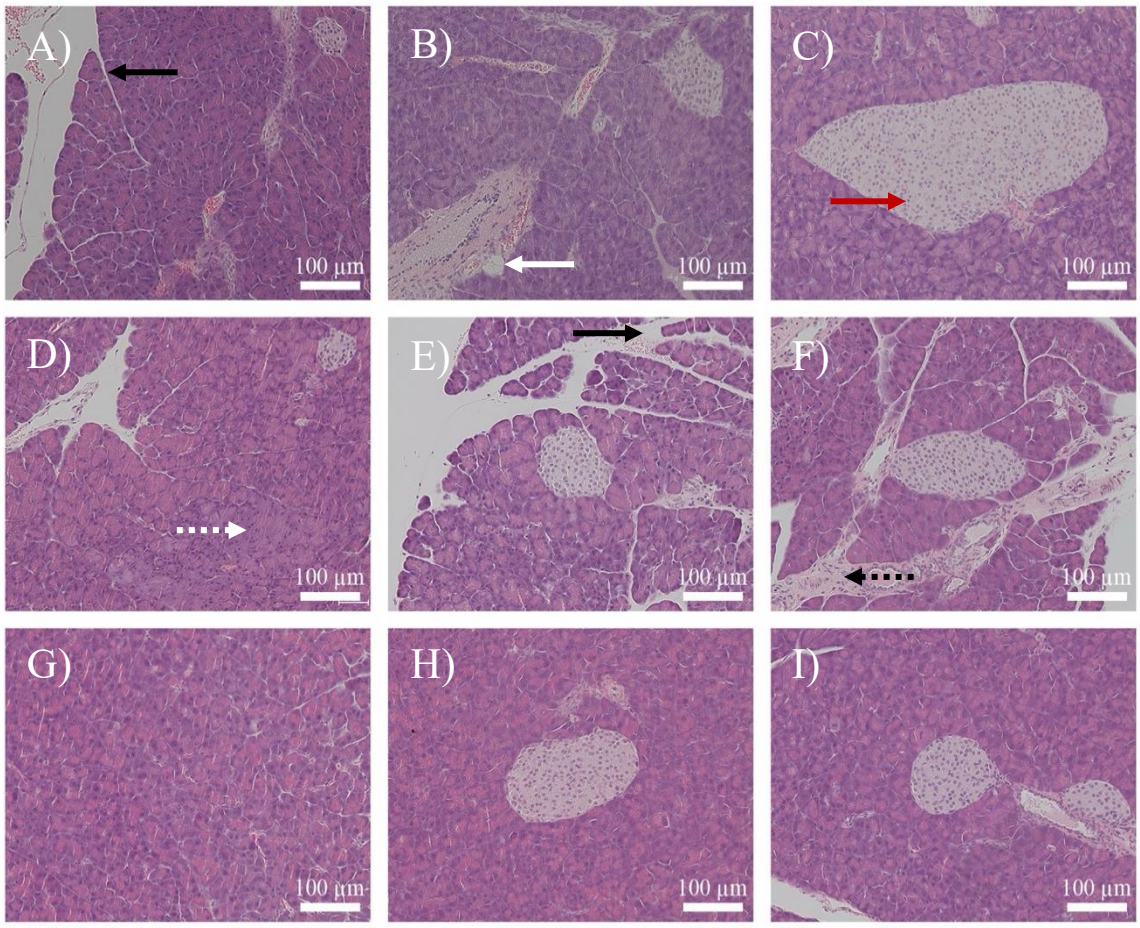

Figure 7: Histology of Male BALB/c, A/J, and C57 Mice Pancreases

Selected H&E histology images of male BALB/c, A/J, and C57 control and 1.5 IU/g peg-asp-treated mice pancreases 5 days post-treatment. No consistent signs of AIP/acute pancreatitis were observed in (A) control BALB/c, (B, C) peg-asp BALB/c, (D) control A/J, (E, F) peg-asp A/J, (G) control C57, or (H, I) peg-asp C57 mice. Pancreatitis or pancreatic disruptive features such as partial edema and inflammation (black solid arrow), pancreatitic fat necrosis (white solid arrow), islet cell hyperplasia (red solid arrow), necrosis (white dashed arrow), and possible leukocyte infilitration (black dashed arrow) were present in peg-asp-treated mice, but some control mice also presented with these pancreatitic markers of damage. Images are scaled to 100 µm.

In the second round of experiments, after A/J mice were selected as the strain to continue to optimize as a potential AIP model, male mice were investigated across multiple timepoints and peg-asp doses, and compared to peg-asp treatment in females. The Dalhousie Histology Core obtained a new Spectra ST + CV partway through the completion of this project, and the new automated staining process versus the earlier manual method created a darker and more concentrated appearance in several samples. The pancreases, however, still did not display a clear AIP profile, and with the use of lower doses, there was less evidence of any type of pancreatic injury. Males injected with 0.750 or 0.375 IU/g peg-asp and dissected 5 days later (**Figure 8A-C**) showed little to no morphological differences from males dissected 3 days post-treatment (**Figure 8D-F**). The most apparent potential interlobial edema is evident in a male 5 days after treatment with 0.750 IU/g peg-asp, as shown in **Figure 8C**. Females, in contrast, at 3-days post-treatment, demonstrated no features of pancreatitis or pancreatitc injury at all (**Figure 8G-I**).

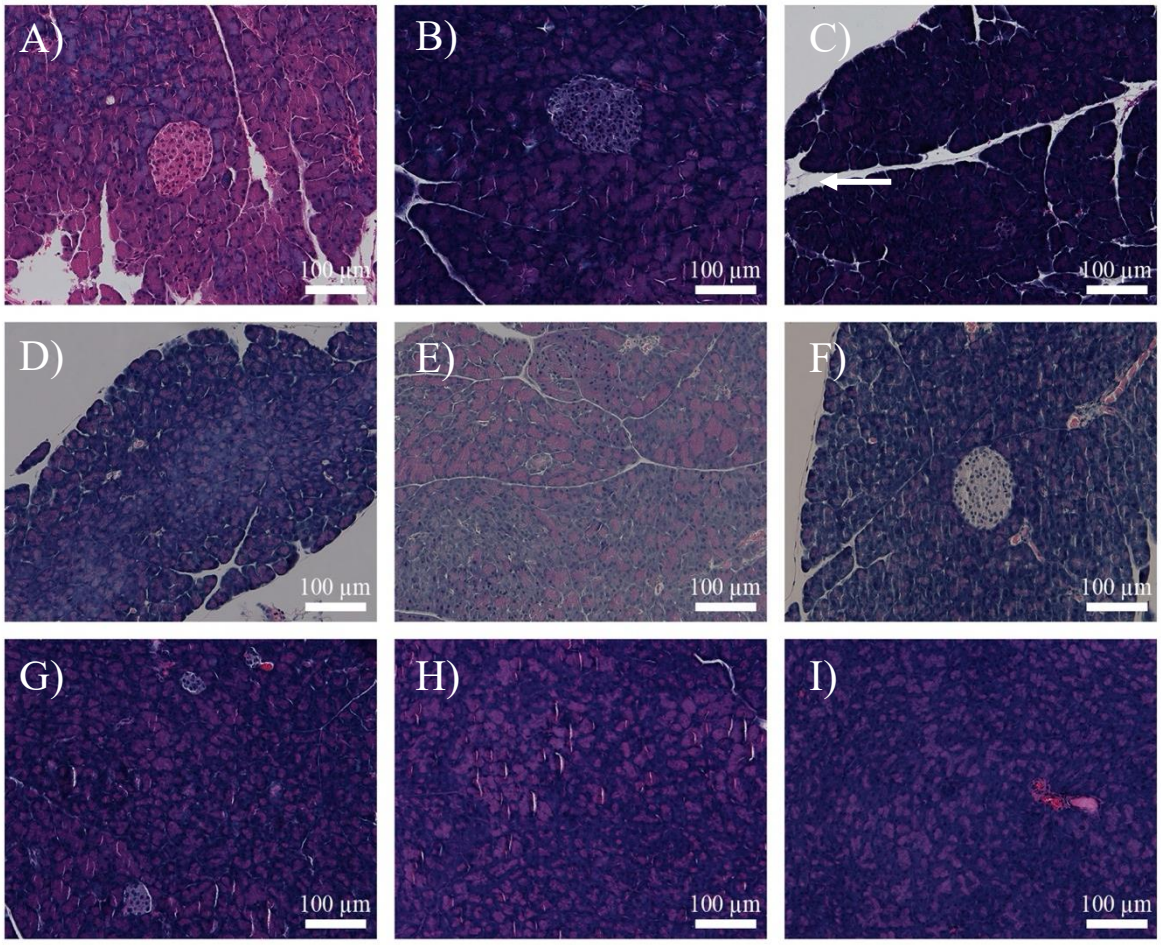

Figure 8: Histology of Male and Female A/J Mice Pancreases

Selected H&E histology images of male and female A/J control and 0.750 or 0.375 IU/g peg-asp-treated mice pancreases 3 or 5 days post-treatment. No consistent signs of AIP/acute pancreatitis were observed in (A) 5-day control male A/J mice, (B) 5-day 0.375 IU/g peg-asp male A/J mice, (C) 5-day 0.750 IU/g peg-asp male A/J mice, (D) 3-day control male A/J mice, (E) 3-day 0.375 IU/g peg-asp male A/J mice, (F) 3-day 0.750 IU/g peg-asp male A/J mice, (G) 3-day control female A/J mice, (H) 3-day 0.375 IU/g peg-asp female A/J mice, or (I) 3-day 0.750 IU/g peg-asp female A/J mice. Mild edema and inflammation is present in panel C (white solid arrow). Images are scaled to 100 µm.

To further probe for possible AIP development, a terminal cardiac blood sample was collected from each A/J mouse in the second experiment and analyzed for total amylase and lipase activity, as an increase of the pancreatic digestive enzymes amylase and lipase 3-times above the upper limit can indicate pancreatitis. Conclusively, serum amylase and lipase across all mice treatment groups remained statistically the same, further indicating that a successful AIP model had not been obtained (**Figure 9A-F**).

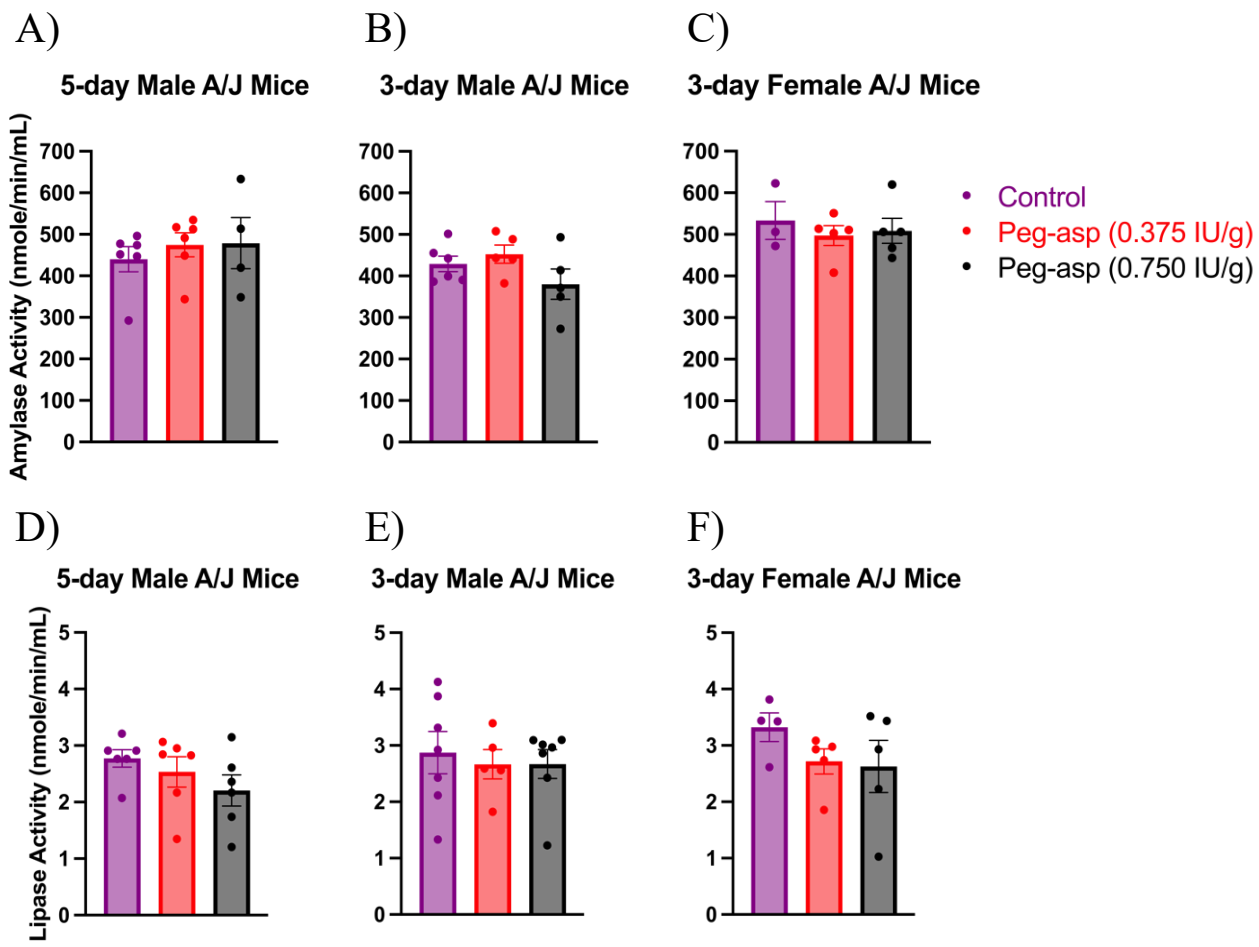

Figure 9: Amylase and Lipase Serum Levels in Male and Female A/J Mice

Amylase serum levels in (A) 5-day male A/J, (B) 3-day male A/J, and (C) 3-day female A/J mice remained stastically the same compared to controls regardless of the peg-asp dose. Lipase serum levels in (D) 5-day male A/J, (E) 3-day male A/J, and (F) 3-day female A/J mice also remained the same across all treatments as analyzed by a one-way ANOVA using the Tukey's multiple comparisons test.

3.1.3 Exploring peg-asp-induced hepatotoxicity in multiple mice strains

Upon dissection of the BALB/c mice, who were tested first, a striking visual difference between the livers of control versus peg-asp-treated mice was noticed. While the controls exhibited the normal appearance of a healthy, deep red-colored liver, the peg-asp-treated mice had livers that were a spotted light pink/beige color (**Figures 10A,B**). After this pattern was observed in the BALB/c mice, it was decided that the livers from the rest of the other strains would also be dissected and H&E stained section prepared. The same light-colored, potentially fatty liver phenotype was observed in peg-asp-treated A/J mice. Some mice had notably enlarged gallbladders, indicating the presence of cholestasis, a condition in which bile is not properly released into the intestines and can be a result of chronic pancreatitis (**Figures 10C,D**)[157]. C57 peg-asp-treated mice, however, appeared to mostly maintain the same liver anatomy as controls, with some exhibiting a lighter red color and miniscule spotting.

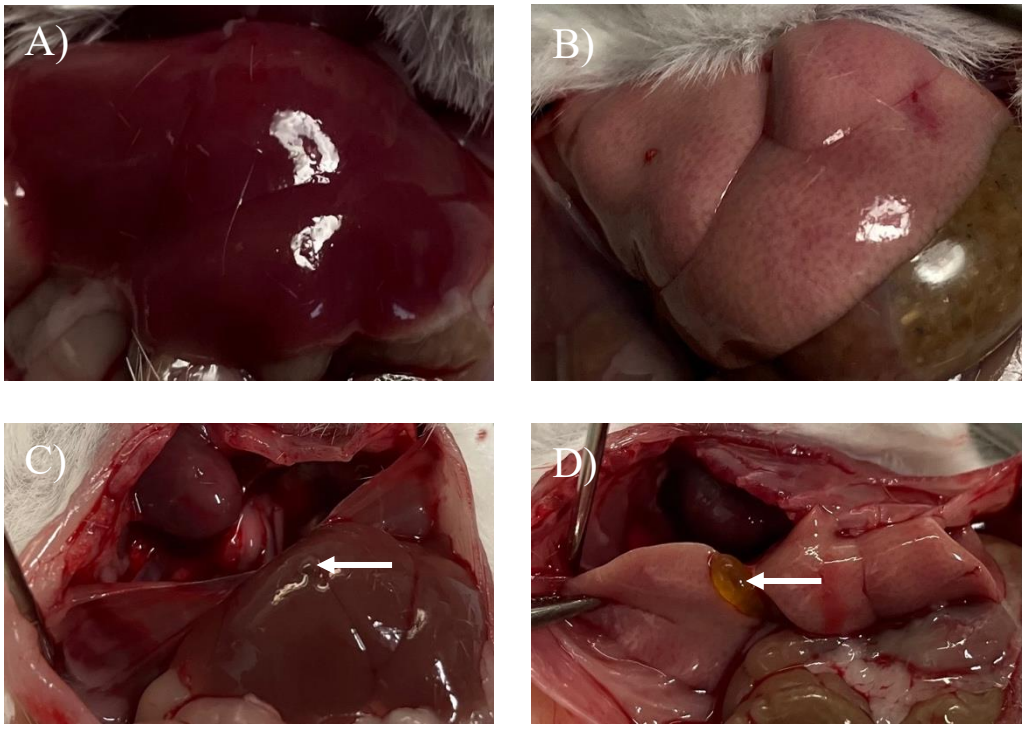

Figure 10: Liver and Gallbladder Gross Anatomy in BALB/c and A/J Mice

The livers of (A) control BALB/c and (B) peg-asp-treated BALB/c mice had clear phenotypic differences from one another, as seen by the healthy red color of the control versus the pink/beige fatty appearance of the peg-asp treated mice. The livers of (C) control A/J and (D) peg-asp-treated A/J mice had a consistently similar pattern to that of the BALB/c mice, with the additional feature of enlarged gallbladders (white solid arrow).

Images of the H&E stained liver samples further attested to the fact that there was a physiological change happening in the peg-asp-treated livers of the A/J mice. There was a noticeable lack of pink eosin staining in peg-asp-treated livers, indicating that the cytoplasm and/or extracellular matrix of the cells had been disrupted (**Figure 11A,B**)[158]. The appearance of white vacuoles that are most likely lipid droplets were also present in the peg-asp samples, suggesting that the mice had developed hepatic steatosis[159]. C57 mice, in contrast, appeared to maintain a more healthy liver appearance (**Figure 11C,D**). However, small lipid droplets can also be seen in the C57 peg-asp treated livers, indicating that the mice were still affected negatively by the drug, though not as dramatically as the A/J mice. Finally, all A/J and C57 livers were weighed, and peg-asp treated A/J livers were found to have a significant reduction in weight compared to same strain controls, while C57 mice liver weights were statistically maintained between the two treatment groups (**Figure 11E,F**). The lighter A/J liver weights could have resulted from a reduced intake of food and subsequent usage of their glycogen stores, and/or the presumable fatty liver formation could have made their liver tissues less dense.

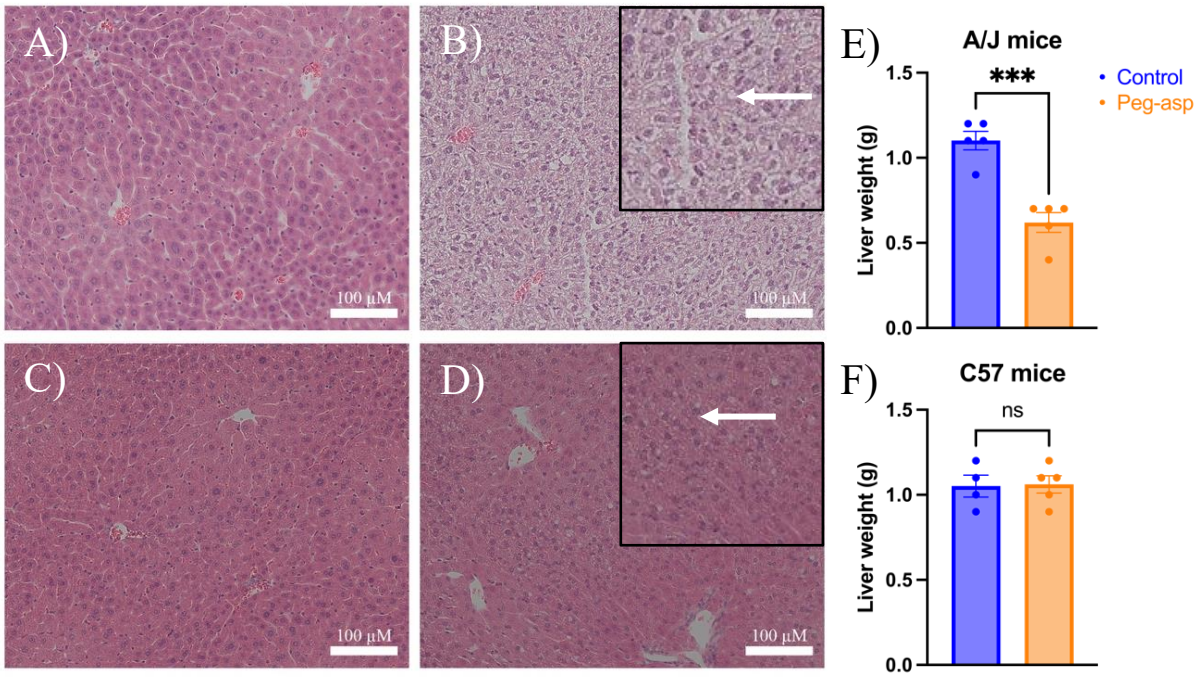

Figure 11: Histology of A/J and C57 Mice Livers and Liver Weights

H&E histology images of (A) control A/J and (B) peg-asp-treated A/J mice revealed differences at the cellular level that further verified the initial gross anatomy observation. Whereas the control livers stained a normal pink color, the peg-asp livers had a diminished uptake of the eosin stain, and had vacuoles that were presumably lipid droplets (white solid arrow, see enlarged panel). (C) Control C57 and (D) 1.5 IU/g peg-asp-treated C57 mice appear to have very similar histological features at first glance, however, there is a clear formation of presumed lipid droplets in the peg-asp-treated mice as seen in the magnified panel. (E) Peg-asp-treated A/J mice liver weights decreased significantly in mass compared to controls, while (F) C57 mice remained the same between both groups. Asterisks (*) indicate a $P \leq 0.05$ as measured by an unpaired t-test.

In the second round of experiments with both male and female A/J mice, fewer histological changes were observed with the use of lower peg-asp doses. Control (**Figure 12A**) and 0.375 IU/g peg-asp-treated male mice (**Figure 12B**) dissected on Day 5 showed few to no histological changes in their livers. On the other hand, Day 5 mice treated with 0.750 IU/g peg-asp (**Figure 12C**) demonstrated clear evidence of lipid droplet formation. However, the lipid droplets had a dimmer, less pronounced appearance similar to those previously seen in the C57 mice at the dose of 1.5 IU/g peg-asp, indicating a dose-dependent hepatotoxic response. Control (**Figure 12D**) and 0.375 IU/g peg-asp-treated male mice (**Figure 12E**) dissected on Day 3 maintained phenotypically healthy livers, while one 0.750 IU/g peg-asp-treated mouse in particular (**Figure 12F**) demonstrated signs of cytoplasmic swelling, similar to a condition known as balloon degeneration, a type of cell death seen in steatohepatitis[160]. Two 0.750 IU/g peg-asp-treated males dissected on Day 3 also had a preliminary hepatic steatosis appearance. Female livers, in contrast, in all treatment groups demonstrated no obvious signs of fatty liver or cell death (**Figures 12G-I**), with the exception of a single 0.375 IU/g peg-asp-treated female, who may have had mild cytoplasmic swelling (not shown).

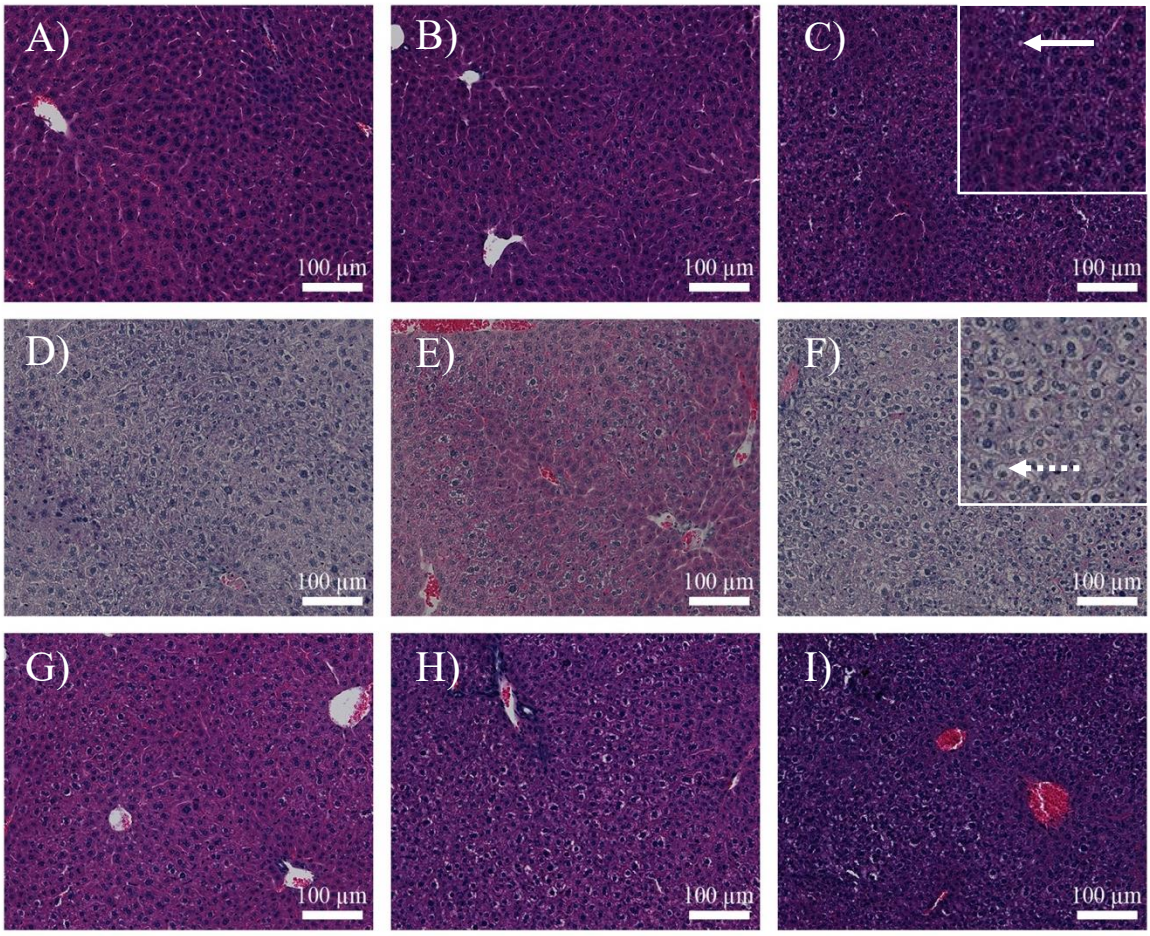

Figure 12: Histology of Male and Female A/J Mice Livers

Selected H&E histology images of male and female A/J control and 0.750 or 0.375 IU/g peg-asptreated mice livers 3 or 5 days post-treatment. No sign of liver injury were present in (A) 5-day control male A/J or (B) 5-day 0.375 IU/g peg-asp male A/J mice, but presumed lipid droplet formation can be seen in (C) 5-day 0.750 IU/g peg-asp male A/J mice (white solid arrow). (D) 3-day control male A/J and (E) 3-day 0.375 IU/g peg-asp male A/J mice are also histologically normal, with (F) 3-day 0.750 IU/g peg-asp male A/J mice exhibiting cytoplasmic swelling similar to balloon degeneration (white dashed arrow). (G) 3-day control female A/J, (H) 3-day 0.375 IU/g peg-asp female A/J, and (I) 3-day 0.750 IU/g peg-asp female A/J mice are all morphologically healthy. Images are scaled to 100 µm.

3.1.4 Evaluating changes in A/J pancreatitic PITX2, RARβ, RARα and ASNS gene expression

The expression of the transcription factor genes of interest, *PITX2* and *RARβ*, in relation to AIP as proposed by the CPNDS, were measured in A/J mice pancreas samples by RT/ qPCR. Additionally, another subtype of the retinoic acid receptor family known as retinoic acid receptor alpha (*RARα*), and the enzyme responsible for synthesizing endogenous asparagine, *ASNS*, were explored for comparison to *RARβ* expression, and investigation of the asparagine depleting effects of peg-asp, respectively. Male A/J mice dissected on Day 3 had the most robust data as they had the most samples that were successfully analyzed in comparison to all three housekeeping genes *GAPDH*, *cyclophilin*, and *RNA poly II*. All genes of interest had n = 4-6, with the exception of 0.375 IU/g peg-asp treated mice in relation to *RARα* that only had an n = 2. For those in which statistical analysis could be performed, the same level of gene expression in *PITX2*, *RARβ*, *RARα*, and *ASNS* across all treatments and timepoints was observed (**Figure 13**). Preliminary genere expression data were obtained for male A/J mice dissected on Day 5 and females on Day 3(n = 1-4), revealed no obvious trends in gene up- or down-regulation.

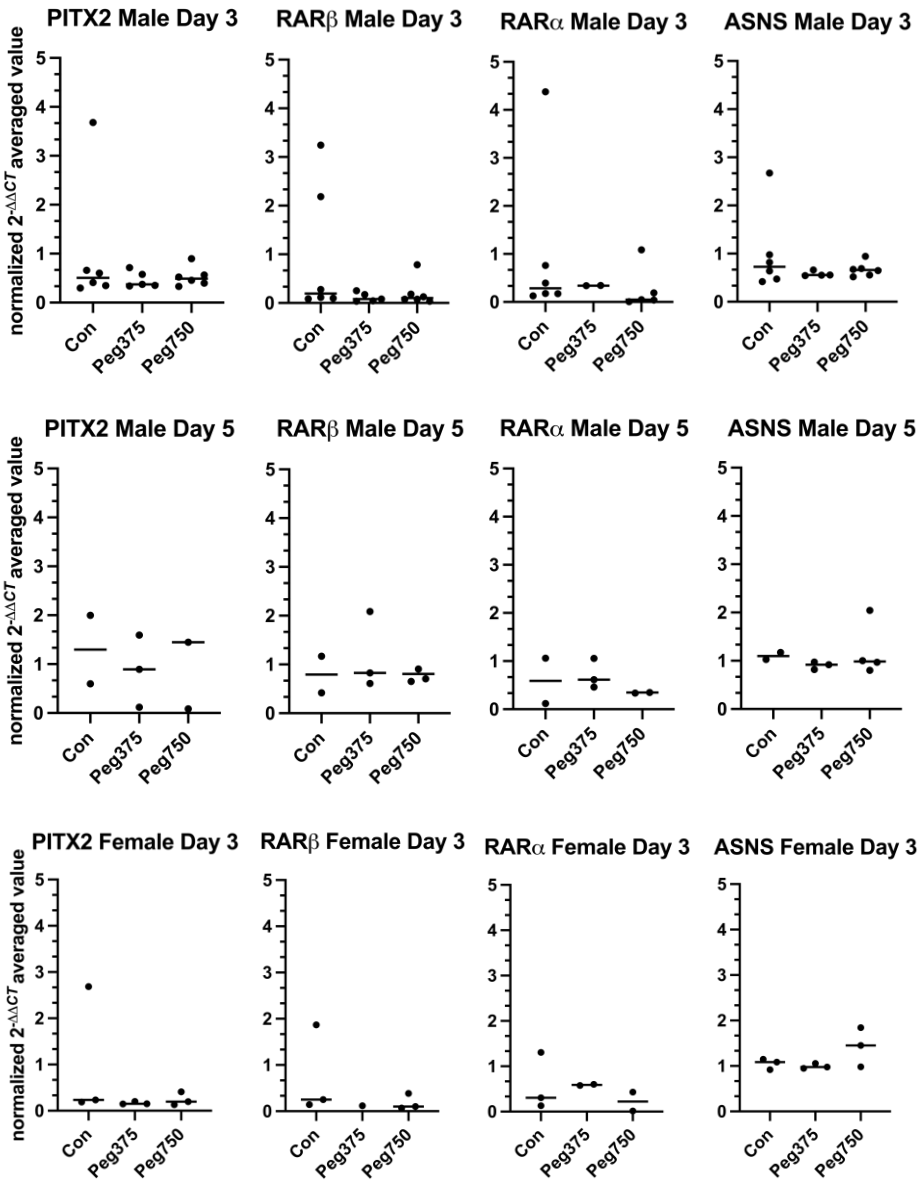

Figure 13: *PITX2*, *RARβ*, *RARα*, and *ASNS* mRNA Pancreas Expression in Male and Female A/J Mice

RT/qPCR analysis was performed on pancreases from male and female A/J mice treated with control PBS or 0.750 or 0.375 IU/g peg-asp. A Kruskal-Wallis test and a one-way ANOVA using the Brown-Forsythe test and Barlett's test found no statistical differences in *PITX2*, *RARβ*, *RARα*, and *ASNS* expression between all male Day 3 treatment groups with full data sets. The male Day 5 and female Day 3 preliminary data also demonstrates no obvious trends.

CHAPTER IV: DISCUSSION

4.1.0: Summary of findings and future directions for optimizing an AIP mouse model

4.1.1 Peg-asp-induced weight loss varies between mice strains and may depend on diet

To our knowledge, this is the first time that peg-asp-associated weight changes have been recorded across multiple strains of mice in a single study. The idea to investigate AIP in multiple mice strains stemmed from another study by Seifert et al. that used taurocholate as the pancreatitis inducing agent[144]. Undoubtably, differences in murine genetics will influence their response to drugs, as was shown by Seifert et al. when the 9 mouse strains tested demonstrated significantly varying pancreas histological scores and amylase and lipase levels from one another[144].

In the first set of experiments, there was no significant weight loss between the BALB/c control and peg-asp-treated groups, which was an unexpected effect considering that asparaginase promotes weight loss in mice, proposedly through metabolic dysregulation independent of reduced food intake[98,99]. However, a lack of weight loss in mice treated with asparaginase has also been described in the literature, as in the case of Tsai et al., when they observed no weight changes in *E. coli*-derived asparaginase-treated C57 mice[127]. In contrast, a significant decrease in total body mass was observed in our peg-asp-treated C57 mice, suggesting that the asparaginase-associated weight loss may be dependent on the asparaginase formulation used, the dose administered, and/or the total number of injections.

A/J mice, which were novelly investigated in the use of asparaginase entirely, showed a considerable decrease in overall weight throughout the duration of the first experiment. In comparison to C57 mice, A/J mice had over twice the amount of total average weight loss at the endpoint when given 1.5 IU/g peg-asp. One confounding factor that could have contributed to

less weight loss in C57 mice is their dietary intake of the supplemental food gel. The three strains were analyzed in sequence (BALB/c, A/J, and C57), and after observing the significant weight loss in the A/J mice, a supplemental food gel was provided to the C57 mice as an animal welfare requirement. The gel was possibly easier to consume than the dry pellets or mash, and thus, the peg-asp-treated C57 mice may have been able to consume more food than they normally would, even in the presence of a decreased appetite. Additionally, if any of the minerals inside of the gel provided an unrecognized protective effect, it could have diminished the weight loss action of asparaginase, and also the subsequently observed hepatotoxicity.

Indeed, diet has recently become a popular area of interest in understanding AIP development. In their 2023 article, Tsai et al. found that patients who developed AIP had less dietary intake of vitamin A than their counterparts who did not develop AIP[127]. Furthermore, circulating vitamin A in the serum of mice was significantly depleted when treated with asparaginase[127]. However, the vitamin A content was only present at a concentration of approximately 0.007 IU/g in the supplemental gel, as opposed to 30 IU/g in the regular chow, so theoretically this could have negatively impacted the C57 mice based on the assessment of Tsai et al. Nonetheless, other ingredients in the gel that have not yet been explored in regards to AIP research could have potentially minimized the ADRs of asparaginase in the C57 mice who had access to it throughout the whole experiment.

As discussed previously, research in mice has also investigated mitigating AIP or pancreatitc injury resulting from asparaginase via the injection and ingestion of L-carnitine and galactose, respectively[99,123]. As the positive protective benefits from these dietary agents has only been investigated as a pre-treatment before a single injection of asparaginase, or only in *ΔGcn2* C57 mice, it would be necessary to explore this further in more clinically translatable scenarios

with the use of repeated asparaginase doses and wildtype mice. Other researchers at Dalhousie University have also recently started to investigate the dietary intake of the main amino acid that asparaginase targets, namely asparagine[162]. Although asparagine is a non-essential amino acid, as it is synthesized endogenously via ASNS, it is also obtained through the diet[163]. Thus, a reduced intake of asparagine may have an impact on asparaginase potency, with less circulating asparagine leading to more severe ADRs. Several types of cells express ASNS throughout the body, but the pancreas is known to be the organ with the highest expression of ASNS, and depletion of circulating amino acids is known to influence the upregulation of pancreatic ASNS[68,164]. The effect that the pancreatitc ASNS upregulation has on the development of pancreatitis specifically, however, remains unclear, and warrants further investigation.

Furthermore, it has been suggested that the established gut microbiota community in an individual before asparaginase treatment could have an impact on circulating asparaginase levels, thus influencing the overall effectiveness of the therapy[165,166]. BALB/c and C57 mice in particular have recently been shown to have significant variation in their representative gut microbial groups between the two strains, and even within the same strain when modifications such as immunodeficiency are introduced[167]. Researchers Guo et al. who conducted this analysis have argued that C57 mice have a more stable genetic background, which could contribute to their more stabilized gut microbiota profile. Less variation in gut microbiota from mouse to mouse is less representative of human variation however, and therefore BALB/c mice may be a better clinical model of choice in this regard[167]. With so many possible scenarios at play, there could be a host of reasons for our observed strain differences in response to asparaginase, from genetics, to diet, to gut microbiota, or a combination of all such factors.

The second set of experiments again revealed an asparaginase-associated weight loss effect in both male and female A/J mice that appeared to be largely dose-dependent. Interestingly, peg-asp-treated females lost less weight in comparison to controls than their male equivalents. It should be acknowledged, however, that the control females also gained more weight than the control males, which could effect the ratio percent loss. The cause for this remains unclear, as both male and female A/J mice gain approximately 6% body weight between 8-10 weeks of age according to The Jackson Labratory, which is the approximate age at which these mice would have been treated[168]. One possibility could be that any reduced food intake as a result of the peg-asp treatment could have caused male mice to lose more overall weight and lean mass compared to females, as this is known observed sex-difference effect in C57 mice[169]. Nonetheless, this would only possibly explain the difference in weight loss as seen in the peg-asp-treated male versus female mice, but still does not address the controls with their difference in overall weight gain. Perhaps the male mice were under more psychological stress for unobserved reasons, and thus, had a decreased rate of weight gain. However, in light of the short duration of the experiment and small sample population, the sex differences observed could also simply be owing to random variation.

4.1.2 Peg-asp-induced hepatotoxicity may be dose-dependant and rely on mice genetics

The primary goal of this study was not successfully obtained in developing an overt AIP mouse model. However, several novel pieces of knowledge were gathered from the experiments that can help direct the next wave of AIP research. First was the unique observation that different strains of mice react differently to asparaginase, as seen in their varying degrees of ADR responses with regards to weight loss and hepatotoxicity. As mentioned heretofore, the ingestion

of the supplemental gel possibly played a role in this effect with the C57 mice, but the BALB/c had no access to the gel, while A/J mice only had access to it for less than 24 hours. Thus, it is necessary to explore the genetic and behavioral differences in the mice to hypothesize what could be occurring at the molecular level.

The first point of interest is that BALB/c mice and A/J mice are both albino strains, unlike C57 mice. As far back as 1967, researcher John Fuller noticed that albino mice appeared to perform less well in certain physical activities such as escaping from water, and generally became more stressed with test procedures in comparison to black C57 mice[170]. Stress in of itself can potentially lead to or worsen the development of liver diseases, and thus could have contributed to the more pronounced hepatotoxicity seen in the BALB/c and A/J mice[171]. BALB/c mice are also known to have increased sensitivity to the hepatotoxin carbon tetrachloride in comparison to C57 mice, which may have likewise been the case as seen in their more dramatic fatty liver appearance when exposed to peg-asp[172]. A/J mice similarily have been shown to have a greater susceptibility to acetaminophen-induced hepatotoxicity compared to C57 mice[173]. However, the type and severity of liver toxicity observed, such as triglyceride and cholesterol accumulation and subsequent release of inflammatory markers, may depend on the drug agent used as well as any diet modifications that could interact with exsiting genetics[174].

Inducing asparaginase-associated hepatotoxicity was not a primary objective of this study, however, it was observed on several occasions, particularly at the highest dose of 1.5 IU/g peg-asp. It was also dose-dependently present in some mice at 0.750 IU/g peg-asp, and only possibly present in a single female A/J mouse treated with 0.375 IU/g. Asparaginase is known to induce different types of hepatotoxicity or neighboring bile duct complications, including hepatic steatosis and choleostasis, and is a well-documented ADR present in both human clinical care

and animal models[175,176]. Furthermore, hepatotoxicity is thought to develop due to a similar reason as to that of AIP, with the depletion of asparagine proposedly causing a decrease in protein synthesis in both organs[133,176]. Interestingly, hepatotoxicity can be treated through dietary interventions including L-carnitine and vitamin B infusion, and a low-fat diet[177].

4.1.3 Peg-asp treatment in mice has to be optimized to stimulate reliable, replicable AIP

Two of the three parameters that are directly used to diagnose pancreatitis in humans, namely, physiological changes of the pancreas, and three-times the normal levels of amylase and lipase, were investigated in parallel in the mice [101,102]. Although the diagnostic marker of abdominal pain was not directly measured, some peg-asp-treated mice did hunch or appear to socially isolate for some time, or have greasy, undergroomed coats, which are all signs of potential physical pain. These symptomatic expressions could have indicated that they were experiencing temporary distress from acute pancreatitis that was not detectable at the terminal endpoint.

With regards to physiological changes of the pancreas, some histological manifestations of a previous AIP episode were present in a few of the peg-asp-treated mice, specifically in both the BALB/c and A/J mice, but not in the C57 mice according to the author's assessment. Dr. Olivia Tsai who performed a blinded analysis of the histological sections in contrast did observe possible AIP in C57 mice as well as the two other strains. However, some control mice also appeared to have some pancreatitic damage, which was an unexpected effect that both assessment parties observed. The reasoning behind this remains unclear, but it is possible that the mice were experiencing stress throughout the duration of the experiment that lead to changes in their pancreas profile.

Finally, in reference to amylase and lipase levels as a means of AIP diagnosis, none of the peg-asp-treated mice in the second round of experiments experienced significant changes in serum levels of pancreatic enzyme. It is important to recall that an increase in amylase and lipase serum levels is also not always observed in other pancreatitis mouse studies or in the human clinic, even in patients diagnosed with acute pancreatitis. Thus, this finding does not rule out the possibility that the mice had developed AIP at some point. However, even with considering the presumable symptomatic pain and pancreatic histological imaging, it is logical to conclude that none of the mice in this study were in a current stage of acute AIP when euthanized and dissected.

For future experiments, it will be important to optimize this potential AIP model through the use of a dose likely somewhere between 0.750 – 1.5 IU/g peg-asp, with evaluation occurring at earlier timepoints, perhaps within several hours of injection. A study that utilized cerulein as the pancreatitis agent found that acute pancreatitis in mice developed rapidly 4 hours post-injection, reached its presumable peak toxicity around 8 hours, and were in a recovery phase by 24 hours[150]. Thus, it is possible that our experiments simply missed the window of optimal AIP, as we did see possible histological suggestions of previous AIP development in some of the pancreases. Based on very recent published literature, injecting mice with IM rather than IP asparaginase may be more appropriate. The paper released by He et al. as seen in **Table 2** was published during the writing of this thesis, after the experiments were conducted[128]. They are the first to have seemingly confidently developed an AIP mouse model in wildtype mice, and they were the first to try the IM injection in this regard. IM and IV are the two routes used to treat humans with asparaginase in the clinic, and thus, will likely be a more accurate representation of asparaginase pharmacokinetics and pharmacodynamics and ADRs when administered this way in

mice[178]. IP additionally poses the risk of exposing the liver the most to asparaginase through absorption into the portal circulation, which IM would avoid with a more gradual, prolonged exposure throughout the body that could result in a more significant influence on pancreatitis development[179]. On the other hand, IP administration has been shown to arguably have better bioavailability than that of IM in rats, so its usefulness may depend upon the goals of the study[180].

4.1.4 PITX2 and RARβ involvement in the development of AIP

Although my findings showed that no statistical differences in the gene expression of pancreatic *PITX2* and *RARβ* were found regardless of the peg-asp dose administered in A/J male Day 3 mice, this study does not rule out their potential involvement in AIP. As discussed in the introduction, *PITX2* plays an essential role in abdominal wall development and is a possible oncogene in pancreatic cancer via inhibition of ferroptosis, a type of iron-dependent, non-apoptotic cell death that is induced by lipid peroxidation[115]. Although its role in AIP is unknown, there are many overlapping similarities between acute and chronic pancreatitis and pancreatitic cancer insomuch that it can be a challenge to distinguish the diseases one from another[181]. Thus, along with the fact that chronic pancreatitis can lead to the development of pancreatitic cancer, it is quite possible that *PITX2* would also be directly involved in advancing AIP[181].

RARβ on the other hand has been shown to play a role in embryonic pancreatic islet cell development and vitamin A-dependent immunity through assisting in immunoglobulin A (IgA) production via B-cells, for example, which serves as a first-line defence mechanism against potential mucosal infections[116,117,182]. Dietary intake of vitamin A is the only source through which humans can obtain this vital micronutrient, and it is converted into retinol and retinoic

acid via the intestinal epithelium[117,183]. Retinoic acid then activates RARs that in turn control other gene expression in the body[117]. As Tsai et al. observed a possible correlation between reduced dietary vitamin A intake and AIP development, as well as reduced circulating and hepatic retinol in mice, the involvement of *RARβ* in AIP is of particular interest to continue researching[127]. If genetic variation and/or asparaginase treatment reduces the function or gene expression of *RARβ*, this could play a similar role to that of vitamin A depletion as protective vitamin A singalling through *RARβ* would be reduced, potentially leading to pancreatitc damage in the form of AIP.

Notably, the use of a higher dose of peg-asp, repeated doses, or a different experimental endpoint could also all factor into capturing an acute or chronic change in *PITX2* and *RARβ* gene expression that was missed in our experiments. We also only measured mRNA content and not that of protein expression, so while the transcriptional expression remained the same, it is possible that changes were happening at the translational level that we did not explore. There were also no pancreatitc expression changes observed in the *RARα* and *ASNS* genes. This was particularly unexpected for *ASNS*, as asparaginase has the potential to cause an upregulation of the enzyme with the depletion of circulating asparagine. However, the mice were only exposed to a single injection of peg-asp, which may not have been significant enough to induce this change, especially considering that the peak window of drug activity was likely not captured at the terminal endpoint.

4.1.5 Other possible interferences and limitations in this study

Other possible factors that interfered with this study and any present limitations are also necessary to consider. In the first set of mouse experiments, all mice were subject to the ongoing additional stressor of saphenous blood vein collections, as the original intention was to analyze

blood serum amylase and lipase levels every second day of the experiment. The serum volumes collected were variable and sometimes altogether unsuccessful, and thus, only the terminal cardiac blood was used in the preliminary and final assessments. This extra handling and any pain associated with needle pricking and brusing of one or both legs could have certainly affected the animal's wellbeing beyond the administration of asparaginase alone.

Moreover, mice in all experiments were fed ad libitum, which is recognizably a potential source of error in regards to weight loss. In the future, control mice should be pair-fed to peg-asp-treated mice so as to account for reduced food intake and subsequent weight effects and other ADRs. However, in an experiment that pair-fed control C57 mice to those treated with asparaginase, weight loss appeared to be independent to that of food intake, suggesting that other currently unidentified metabolic factors may be at play [98].

Nonetheless, if peg-asp-treated mice were eating less in our experiments, this could have affected their amylase and lipase levels in a way that was masked without the control of pair-feeding. If the control mice were eating right before dissection, this could have caused a digestion-induced increase in amylase and/or lipase activity levels that could have been comparatively close to a possible peg-asp-treated abnormal elevation. Thus, we may not have properly observed baseline levels of amylase and lipase activity in the control mice, which made the results appear as if there was no significant difference compared to peg-asp-treated mice when there could have been. To account for this, all mice could be fasted for a humane 6 hours pre-euthanization to normalize dietary effects on serum pancreatic levels. In the future, all mice should also have the same access to the same food sources throughout the experiment, including the pellets, mash, and supplemental gel, to reduce the presence of diet-dependent variability.

Further limitations that could have impacted this study include that the histological samples were only analyzed via H&E staining. In the future, immunohistochemistry could be used to probe for activated macrophages and pancreatic stellate cells that could be indicative of pancreatitis development[184]. Immunohistochemistry could also be used to observe the protein products of the *PITX2* and *RARβ* genes of interest in the pancreas tissue samples. With regards to the livers, it would be informative to use oil red O staining to better visualize and identify lipid formation as an indicator of hepatosteatosis[185].

Finally, we did not directly monitor the blood concentrations of peg-asp throughout the experiment, which would possibly correlate with the severity of ADR outcomes, but recommend doing so in future experiments. Baseline asparagine levels could also be determined and asparagine depletion could be tracked throughout the duration of the experiment to monitor activity levels of peg-asp. One could also measure glutamine depletion, or even the resulting by-product levels of peg-asparaginase activity, namely, aspartic acid, glutamic acid, and ammonia, to directly quantify peg-asp activity in real time.

4.2.0: Conclusion

In summary, this work has shown for the first time that there are mice strain-dependent differences in the response to asparaginase treatment, as seen in the weight loss and hepatotoxicity variabilities. Although A/J mice were selected as the strain to move forward with as they had the most severe reaction to asparaginase, it is important to recognize that the other strains may also provide great insight into the furthering of AIP research. The BALB/c mice may be of particular interest as they developed serious liver toxicity but did not experience significant

weight loss. They have also demonstrated the favorable quality of having more diverse gut microbiota compared to C57 mice that could contribute to influencing the development of AIP.

Optimization of the asparaginase formulation, administration route, dosage concentration, number of doses, terminal timepoint, and diet are all factors to consider in further developing a reliable AIP mouse model. Other strains of mice not explored here could also be of potential interest to investigate as they may present with other responses to asparaginase at the genetic level that may be missed when focusing on a single strain. Future researchers should also continue to investigate the role of *PITX2* and *RARβ* in the eventual AIP model, and also take into consideration other possible genes of interest that may be involved in pancreatitis development. Ongoing work lead by the CPNDS may provide such insight. Overall, we are closer than ever to understanding the mechanisms of AIP development, and this work has provided some insight into the logical next steps.

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*Note: Figures 2, 3, and 4 were created with BioRender.com.