7 DISCUSSION

7.1 Summary of Findings

Although the I148M variant in PNPLA3 was identified as a major risk factor for NAFLD progression over a decade ago, our understanding of the function of PNPLA3 in health and disease remains largely obscure. The controversy surrounding the function and pathogenesis of PNPLA3 stems largely from the lack of an appropriate human model that expresses endogenous levels of the protein.

In this thesis, we used CRISPR/CAS9 and hiPSCs to create a novel, *in vitro*, human model to study the effects of *PNPLA3* genotype on NAFLD development and progression. We used CRISPR/CAS9 to create genetically edited hiPSC lines that were homozygous for the reference allele ($PNPLA3^{UC}$), homozygous for the risk allele ($PNPLA3^{I148M}$), or had a complete knock-out of the PNPLA3 gene ($PNPLA3^{KO}$) in two different genetic backgrounds (FSPS13B and A1ATDR/R). Following the generation and validation of these lines, we differentiated each line into HLCs using an established protocol that mimics the embryonic development of hepatocytes. We demonstrated that PNPLA3 genotype does not affect the capacity of hiPSCs to differentiate into HLCs. We also examined the effect of 3D culture on these HLCs and found that this culture method improved their maturity. Therefore, for all NAFLD modelling experiments, differentiated HLCs of each genotype were cultured in this 3D format.

In order to examine the effect of *PNPLA3* genotype on lipid metabolism, we treated the lines with control medium, to model a normal diet, or medium supplemented with oleic acid or palmitic acid to model the NAFLD disease phenotypes of steatosis and lipotoxicity, respectively. We found that regardless of treatment, there was a stepwise increased in lipid accumulation from $PNPLA3^{UC}$ cells to $PNPLA3^{I148M}$ cells to $PNPLA3^{KO}$ cells. In addition to this increased steatosis, PNPLA3-edited cells were also resistant to lipid-induced stress. $PNPLA3^{I148M}$ cells and $PNPLA3^{KO}$ cells failed to upregulate ER stress markers in response to palmitic acid treatment and these PNPLA3-edited cells were resistant to the lipotoxic effects of this SFA. This resistance to

lipotoxicity gave way to a steatotic phenotype that closely resembled oleic acid treated cells.

In order to elucidate the mechanisms behind this increased steatosis and resistance to lipotoxicity, we used lipidomic and transcriptomic data from each of the lines. We found that PNPLA3^{I148M} and PNPLA3^{KO} cells had vastly different lipidomic profiles from PNPLA3^{UC} cells. The PNPLA3-edited cells accumulated higher concentrations of triglycerides and these triglycerides were preferentially composed of PUFAs and SFAs. These lipidomic findings offered insight into the functionality of PNPLA3 as well as the mechanism by which these cells escape from SFA-induced lipotoxicity. PNPLA3 has been hypothesized to have lipid droplet remodelling functionality. Specifically, it is hypothesized that PNPLA3 catalyses the transfer of PUFAs from triglycerides to phospholipids and the I148M variant results in the loss of this functionality. The accumulation of PUFA species in the triglycerides of PNPLA3-edited cells in our system supports this hypothesis. Additionally, the accumulation of SFAs and PUFAs in triglycerides has been proven to be cytoprotective against SFA-induced lipotoxicity, offering insight into the phenotypic differences between genotypes. In order to test the hypothesis that PNPLA3-edited cells escape SFA-induced lipotoxicity by diverting SFAs from metabolic pathways into triglyceride storage, we used small molecule inhibitors to block triglyceride formation in our system. In support of this hypothesis, we found that blocking triglyceride formation in our system re-sensitized PNPLA3-edited cells to SFA-induced lipotoxicity.

Given these data, we hypothesized that loss of PNPLA3 function results in the downregulation of lipid catabolism pathways in favour of triglyceride formation. RNA sequencing of the three genotypes revealed a global downregulation of lipid metabolism pathways involved in the homeostasis of fatty acids, triglycerides, cholesterol, and phospholipids. This analysis also revealed that, perhaps in part due to their metabolic dysregulation, PNPLA3-edited cells fail to metabolically differentiate between oleic acid and palmitic acid. This finding offers further evidence in support of the hypothesis that PNPLA3-edited cells avoid lipotoxicity by downregulating metabolic pathways and preferentially storing all fatty acid species as triglycerides.

In addition to the downregulation of lipid metabolic pathways, PNPLA3-edited cells also downregulated the expression of DMEs in every phase of the drug metabolism cascade. Given this global downregulation and the clinical evidence that the I148M variant plays a role in progression of diseases of drug metabolism such as ALD, we hypothesized that loss of PNPLA3 function makes hepatocytes more susceptible to hepatotoxicity. In support of this hypothesis, we found that $PNPLA3^{KO}$ cells were more susceptible to ethanol-induced toxicity than $PNPLA3^{UC}$ cells, even at concentration that was sublethal for the $PNPLA3^{UC}$ cells. However, we failed to replicate this increased lethality in two other models of hepatotoxicity, iron and acetaminophen. Therefore, additional studies are needed to elucidate the role of PNPLA3 in drug metabolism and hepatotoxicity.

In nearly every experiment that was performed, the $PNPLA3^{I148M}$ cells represented an intermediate phenotype between $PNPLA3^{UC}$ and $PNPLA3^{KO}$ cells. These results demonstrate that the I148M variant in PNPLA3 is a loss of function variant. The most compelling evidence for this finding is the RNA sequencing data that showed in both principle component analysis and heatmap analysis that $PNPLA3^{I148M}$ cells represented a near perfect transcriptomic intermediate between $PNPLA3^{UC}$ and $PNPLA3^{KO}$ cells. This finding represents the first conclusive evidence from a human experimental system that the I148M variant is a loss of function variant.

Our findings are summarized in Figure 7.1. This study represents the first step in fully elucidating the role of PNPLA3 and its deficiencies in the pathophysiology of NAFLD. Our system has the advantage of being derived from human cells and demonstrating normal PNPLA3 expression patterns and levels. The use of CRISPR/CAS9 allowed us to create isogenic lines of three genotypes to not only explore the mechanistic effects of altered *PNPLA3* genotype on cell function but also to answer the most controversial question in the field: is the I148M variant a loss of function or gain of function variant? The use of hiPSCs to create this model offers the opportunity to explore the effects of *PNPLA3* genotype on the functionality of several different hepatic cell types that play a role in NAFLD development and progression. The work in this thesis has offered new insight into the functionality of PNPLA3 and its pathophysiology in NAFLD. Our system was used to validate clinical findings and identify the hypotheses from previous

publications that are most applicable to a human model of PNPLA3-induced NAFLD. However, a great deal about the function of PNPLA3 in healthy tissue and disease remains obscure, so additional studies are needed. We believe that the *in vitro* system developed in this thesis represents the ideal platform in which to elucidate the full pathophysiology of the PNPLA3 I148M variant in NAFLD development and progression.

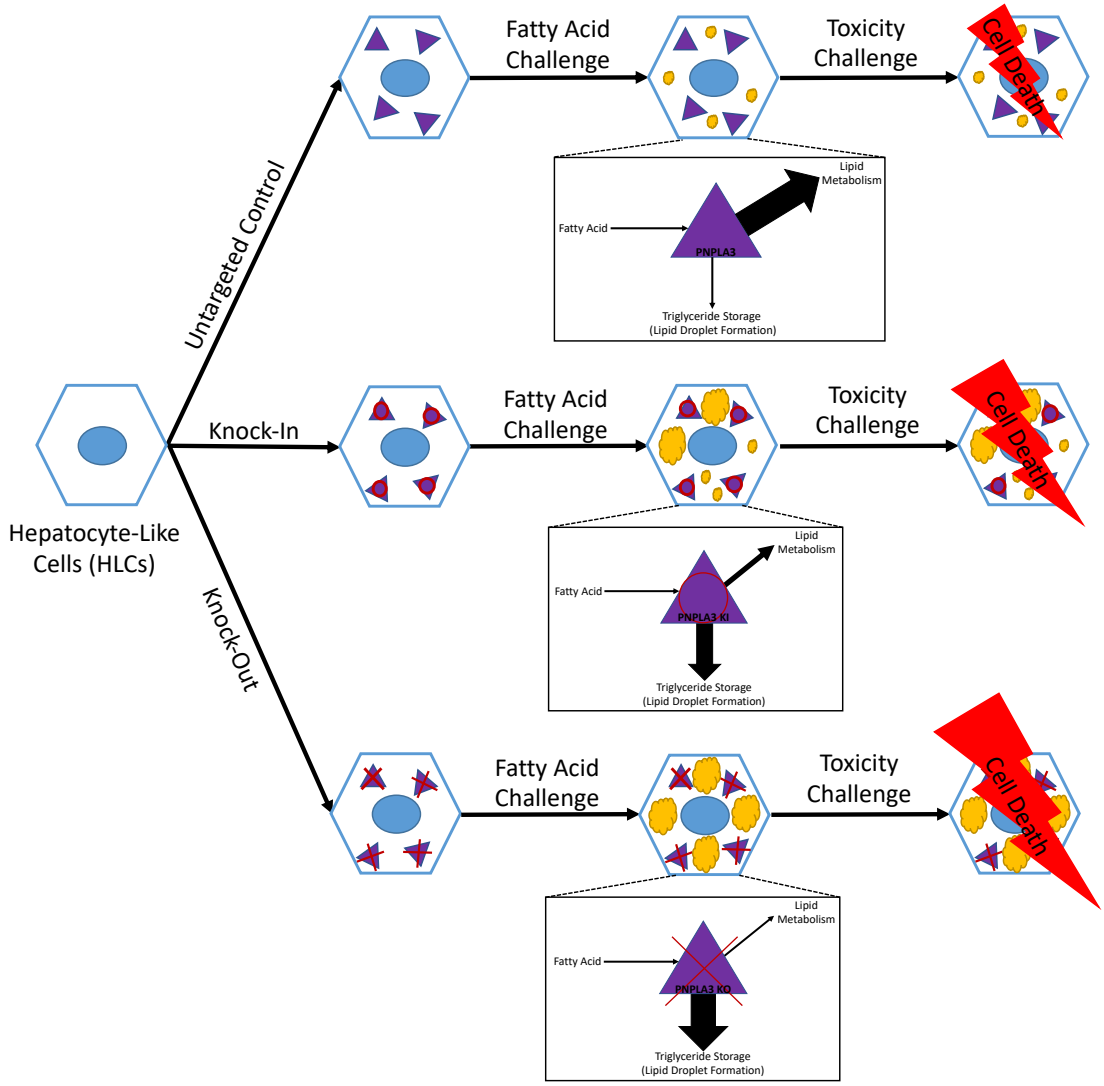

Figure 7.1 Summary of findings.

The three *PNPLA3* genotypes exhibited differential phenotypes when subjected to either a fatty acid or a toxicity challenge. When presented with a fatty acid challenge, PNPLA3^{UC} cells upregulate lipid metabolism pathways and few of the fatty acids are esterified into triglycerides. PNPLA3^{KO} cells demonstrate the opposite phenotype downregulating lipid metabolism and storing the majority of fatty acids in triglycerides in lipid droplets. This metabolic dysfunction causes PNPLA3^{KO} cells to be more susceptible to toxic injury than PNPLA3^{UC} cells. In both insult systems, PNPLA3^{I148M} cells have an intermediate phenotype which indicates that the I148M variant is a loss of function variant.

7.2 PNPLA3 is a Lipid Droplet Remodelling Protein

Our data supports the hypothesis that PNPLA3 functions as a lipid droplet remodelling protein and that I148M variant is a loss of function variant. It is clear from the lipidomics data that loss of PNPLA3 function results in the sequestration of certain fatty acid moieties, namely PUFAs and SFAs, in triglycerides. This finding in combination with the RNA sequencing data that indicated a global downregulation of lipid catabolism pathways indicates that the lack of lipid droplet remodelling in PNPLA3-edited cells causes the sequestration of fatty acids in metabolically inert triglycerides.

Lipid droplet remodelling is defined as the act of altering the lipid content within the lipid droplet or the composition of the organelle's membrane in order to influence the protein composition of the droplet's surface. Lipid droplets are extremely dynamic structures and the proteins that coat the surfaces of these droplets vary based upon the metabolic needs of the cell. In the fed state, lipid droplets are remodelled to allow for expansion of triglyceride storage and budding. Whereas in the fasted state, lipid droplets are remodelled to recruit lipases and other enzymes to assist in hydrolysis of triglycerides and transport of free fatty acids to metabolic pathways for energy utilization [31, 33, 248]. Loss of PNPLA3 appears to reduce lipid droplet remodelling especially with respect to transfer of PUFAs from triglycerides to phospholipids. By failing to properly regulate the phospholipid composition of the lipid droplet membrane, the protein composition of the droplet's surface may be altered which may prevent the cell from balancing the rates of triglyceride synthesis and lipolysis [128, 163, 164]. Inhibition of lipid droplet remodelling capacity could result in the sequestration of fatty acid moieties in lipid droplets by restricting the hydrolysis of triglycerides. In this way, loss of lipid droplet remodelling function in PNPLA3-edited cells also explains the downregulation of lipid catabolism genes. By requisitioning fatty acids to triglycerides in lipid droplets, these molecules are restricted from entering other metabolic processes. The diminished supply of lipid precursors leads to a global downregulation of metabolic pathways.

The role of PNPLA3 in lipid droplet remodelling has been well established in both clinical and experimental models [125-127, 165]. This is compelling evidence that validates our model as an appropriate system to study the function of PNPLA3 in health and disease.

The function of PNPLA3 has long been controversial because the protein appears to have a wide range of enzymatic functions; PNPLA3 has been hypothesized to function as a lipase, an acyl transferase, and a facilitator of VLDL secretion [109, 118, 121, 122]. Lipid droplet remodelling is a hypothesis that allows us to reconcile this diverse functionality. This hypothesis contends that PNPLA3 catalyses the lipolysis of triglycerides in order to transfer PUFAs to phospholipids. Therefore, PNPLA3 must utilize both its lipase and acyl transferase capacities to facilitate this lipid droplet remodelling in a PUFA direction. Additionally, by controlling the lipid droplet membrane composition, PNPLA3 influences the formation and secretion of VLDLs. Therefore, the findings from our study are in accordance with established findings on the function of PNPLA3 and offer clarity on the role of this enzyme in lipid homeostasis.

7.3 I148M Variant is Loss of Function

Extensive debate remains about the role of the I148M variant as a loss of function or a gain of function variant. This controversy is compounded by the lack of consensus on the function of the protein. Generally, *in vitro* studies using purified PNPLA3 protein indicate that the I148M variant causes a stark reduction in triglyceride lipase and acylglycerol transacylase activities as well as reduced VLDL secretion [109, 121, 122]. These data strongly suggest that the I148M variant is a loss of function variant. However, when the Pnpla3 protein was knocked out in mice, there was no NAFLD-like phenotype observed [160, 161]. Only when the I148M variant was over-expressed and/or knocked-in to the mouse liver did steatosis occur [123, 163]. Leading many to assert that the I148M variant in PNPLA3 was in fact a gain of function variant. While others still claim that the I148M variant is a dominant negative variant due to the accumulation of I148M murine Pnpla3 on the surfaces of lipid droplets [162, 164]. We assert, based upon the results of our model as well as the preponderance of clinical data, that the I148M variant is a simple loss of function variant.

In our experimental model, the phenotype of the PNPLA3^{I148M} cells unfailingly trended in the same direction as the PNPLA3^{KO} cells. Both of the PNPLA3-edited lines accumulated more lipid droplets than PNPLA3^{UC} cells regardless of treatment type, were resistant to palmitic acid-induced ER stress and lipotoxicity, preferentially accumulated

triglycerides containing PUFAs and SFAs, failed to metabolically differentiate between oleic acid and palmitic acid, downregulated lipid metabolic pathways in spite of lipid stimuli, and showed increased susceptibility to ethanol-induced toxicity. Additionally, the transcriptomic analysis of the three genotypes indicates that PNPLA3^{I148M} cells have a gene expression profile that is functionally midway between PNPLA3^{UC} and PNPLA3^{KO} cells. It is clear from these experiments that the I148M variant does not result in a complete loss of PNPLA3 function. We found that the phenotype of PNPLA3^{I148M} cells varies slightly between cell line, genetic background, and HLC differentiation, in some instances more closely resembling either the PNPLA3^{UC} or PNPLA3^{KO} cells. We believe that these variations can be attributed to variable functionality of the PNPLA3 protein. When the PNPLA3 protein has higher functionality, due to a variety of factors such as genetic background and maturity, it more closely resembles the PNPLA3^{UC} phenotype and vice versa. This result is not entirely unexpected since *in vitro* studies of the purified PNPLA3 protein have shown that the I148M variant retains some PNPLA3 functionality. The variant does not disrupt the catalytic serine and thus retains the theoretical ability to catalyse the hydrolysis of glycerolipids. However, the bulkier side chain of methionine reduces access of the substrate to the active site of the enzyme leading to a reduced, but incomplete loss of enzymatic activity. In other words, the V_{max} of the I148M PNPLA3 enzyme is reduced by 100-fold while the K_m remains unchanged [120, 121]. Since the I148M variant retains the enzymatic capacity to catalyse lipolysis, it is possible that the I148M variant may cause a hypomorphic-type phenotype rather than a complete deletion. Thus, the I148M variant has a phenotype that is intermediate between the reference allele and a complete knock-out. These data offer strong evidence that the I148M variant disrupts the functionality of the PNPLA3 enzyme and causes disease via a simple loss of function mechanism in hepatocytes. This loss of function contributes to NAFLD by inducing steatosis through impaired lipid droplet remodelling that results in downregulation of lipid metabolic pathways and increased susceptibility to certain hepatotoxins such as ethanol.

Our data is supported by clinical findings about PNPLA3 and the I148M variant in patients with NAFLD. The original GWAS study by Romeo et al. found that patients with the I148M variant have increased hepatic fat content. In addition, the authors identified three patients with mutations in the *PNPLA3* gene that were likely to be null. These

patients with null *PNPLA3* alleles were also more likely to have very high levels of hepatic triglyceride content [91]. This indicates that complete loss of PNPLA3 function results in a phenotype closely resembling the I148M variant with increased hepatic steatosis.

As seen in our system, carriers of the PNPLA3 I148M variant retain PUFAs in triglycerides in their livers. Carriers of the risk allele have lower circulating levels of PUFA-containing TAGs while liver biopsies reveal that these patients have increased hepatic levels of TAGs containing 3-11 double bonds. This finding was confirmed in an *in vitro* system using human A431 cells with either a complete knock-out of the PNPLA3 protein or with the I148M variant knocked-in. The A431 I148M and knock-out cells had increased lipid droplet formation and preferential sequestration of PUFAs to triglycerides as well as impaired transfer of PUFAs from triglycerides to phospholipids [124, 165]. These data indicate that in human cells, the I148M variant metabolically resembles a complete knock-out of the gene. The metabolomic phenotype of human PNPLA3 I148M cells also closely resembles that of obligate loss of function Pnpla3 S47A mice. Pnpla3 S47A mice have no enzymatic activity due to mutations in the active site of the enzyme. In mice, the S47A variant results in increased levels of long chain and very long chain PUFAs in hepatic triglycerides while the I148M variant causes the opposite trend [127]. These data indicate that the human I148M variant results in a phenotype that closely resembles both human and mouse loss of function, arguing not only that the I148M variant is a loss of function variant but that the mouse is not an appropriate model to study the PNPLA3 I148M variant.

Finally, those who believe that the I148M variant is a gain of function variant claim that reducing the levels of PNPLA3 expression may improve NAFLD in patients. However, studies of *PNPLA3* SNPs that modulate transcription levels of the protein do not support this hypothesis. The minor allele of the *PNPLA3* SNP rs139051 causes reduced mRNA expression levels of *PNPLA3*; however, this lower expression is not significantly associated with improvement in steatosis, steatohepatitis, or fibrosis. This indicates that the pathogenicity of the I148M variant is independent of transcriptional variability [274]. One study did identify the rs2294918 SNP in *PNPLA3*, which is associated with lower

expression of PNPLA3 and a slight improvement in NAFLD-associated traits in carriers of the I148M variant. However, this study also found that in reducing the expression of the I148I PNPLA3 enzyme, the rs2294918 SNP also reduced the protective effect of the reference allele against NAFLD development and progression [275]. This implies that reduced expression of the PNPLA3 protein predisposes patients to NAFLD offering further evidence that reduced PNPLA3 expression and/or functionality is pathogenic. In all, clinical evidence and *in vitro* studies on human cells indicate that loss of PNPLA3 either through null mutations, reduced expression levels, or the I148M variant contributes to NAFLD development.

Though the clinical data strongly supports the hypothesis that the I148M variant is a loss of function variant, the data from murine models has introduced substantial confusion on this topic. Current consensus in the murine research community is that the I148M variant in PNPLA3 is not a simple loss of function variant but requires the presence of a non-functional protein [162, 164]. This notion is based on the finding that complete knock-out of Pnpla3 in mice does not result in a NAFLD-like phenotype [160, 161]. However, this finding fails to account for the substantial sequence divergence of mouse and human PNPLA3 genes and differences in tissue expression of PNPLA3 between mice and humans. Pnpla3 is endogenously expressed at extremely low levels in mouse livers and high levels in adipose tissue [105, 111, 119]. However, it has been shown that Pnpla3 must be (over)expressed in liver tissue for a phenotype to be observed [123]. Fundamentally, the differences in tissue localization as well as the distinct nutritional regulation between the mouse and human PNPLA3 suggests that this protein may have a different function in these two species. Therefore, we contend that mice are not a suitable model system in which to study the function of PNPLA3 and the pathogenicity of the I148M variant. Despite these profound interspecies differences, we contend that data from the murine models does support the hypothesis that the I148M variant is a loss of function variant, or at the very least, a dominant negative variant. The data from mouse models shows that overexpression of a catalytically dead version of Pnpla3 results in a similar phenotype as overexpression of the I148M variant [109, 163, 165]. These data indicate that the I148M variant is equivalent to loss of Pnpla3 catalytic function. Murine studies contend that steatosis in I148M and S47A mice is caused by accumulation of

mutant protein on the surface of lipid droplets which would make the I148M variant a dominant negative variant [162, 164].

The data from our model does not contradict the dominant negative hypothesis wherein the presence of mutated protein exacerbates the negative phenotype caused by the loss of protein function. We did not observe increased expression of I148M protein in our system, but we did not perform intracellular staining, so it remains possible that this accumulation also occurs in our system. Additionally, the lipidomic data from our model did indicate that PNPLA3^{I148M} cells accumulate more PUFA-containing triglyceride species than even PNPLA3^{KO} cells. This data suggests that the presence of the I148M protein may exacerbate this phenotype by further restricting access of lipid droplet proteins or reducing the mobility of lipids from lipid droplets. Thus, it is possible that the presence of the I148M protein can augment the negative effect of loss of PNPLA3 catalytic function. However, the other experiments in our system indicate that complete loss of PNPLA3 function through genetic elimination causes a more extreme disease phenotype that the I148M variant. Thus, in contrast to murine models, lack of PNPLA3 protein is sufficient to cause a steatotic phenotype which implies that the main pathological effects of the I148M variant are due to a simple loss of catalytic function.

7.4 Our Model and Human Disease

NAFLD can be divided into two related but pathologically distinct features, steatosis and hepatocellular damage leading to inflammation and fibrosis. The I148M variant in PNPLA3 has been conclusively linked to both aspects of NAFLD [91, 132, 133, 135]. In our system, we have been able to clearly demonstrate the role that loss of PNPLA3 function plays in the increased accumulation of intracellular lipid droplet in hepatocytes. However, the other data from our system, namely escape from palmitic acid-induced lipotoxicity and the downregulation of innate immune response and inflammation pathways, are difficult to reconcile with the role of the I148M variant in NAFLD progression. Our data seems to argue that the I148M variant in hepatocytes may cause hepatic steatosis, but the variant may be protective against the hepatocellular damage which is a hallmark of NAFLD progression. We assert that there are several hypotheses that could help explain this seemingly paradoxical result. First, we hypothesize that the

global downregulation of cellular metabolism in hepatocytes with the I148M variant may make these cells more susceptible to hepatotoxicity. Data from our system supports this hypothesis; however, additional studies are needed to confirm its validity. Second, we hypothesize that the decreased lipotoxicity observed in our system may be the *in vitro* manifestation of intact insulin signalling. Finally, we hypothesize that hepatocytes and stellate cells are differentially affected by the I148M variant with hepatocytes manifesting the steatosis and HSCs causing hepatocellular damage, inflammation, and fibrosis. Each of these hypotheses will be discussed at length in the following sections.

7.4.1 PNPLA3 Edited Cells are More Susceptible to Other Forms of Hepatotoxicity

NAFLD is an extremely complex, multifactorial disease and the temporal order of insults has long been up for debate. For decades, the two-hit hypothesis drove NAFLD research asserting that steatosis was the "first hit" that sensitized the liver to a "second hit" of oxidative stress or mitochondrial dysfunction that caused inflammation [276, 277]. Recently, researchers have begun to discount this hypothesis as too simplistic. Nothing in biology can be explained by a simple two-step process. Researchers now accept the slightly more complex, though largely similar, multi-hit hypothesis [63, 278]. Simply, this hypothesis asserts that genetics, epigenetics, environmental factors, and a fair amount of bad luck combine to increase the susceptibility of a patient to progressive disease. The multi-hit hypothesis acknowledges that liver disease does not happen in a vacuum. The liver is constantly bombarded with insults as it is one of the body's first defences against toxic substances whether they originate from endogenous or exogenous sources. It has been well established that patients with NAFLD are more susceptible to drug induced liver injury because the hepatocytes of these individuals are damaged and preoccupied with lipotoxic insults from overwhelming lipid accumulation within the liver [262, 265, 267]. In the spirit of the multi-hit hypothesis, we chose to examine the effect of *PNPLA3* genotype and steatosis on drug metabolism in our system.

The I148M variant has been extensively correlated with increased disease severity for nearly every known chronic liver disease from NAFLD to ALD to viral hepatitis to hereditary hemochromatosis [91, 135, 139, 141, 144]. In addition, this disease variant has also been linked to drug induced liver damage in paediatric patients undergoing

chemotherapy for acute lymphoblastic leukaemia [224, 225]. The contribution of this variant to the pathogenesis of this diverse list of diseases indicates that PNPLA3 plays a role in more than just steatosis progression. We hypothesize that the metabolic dysregulation caused by loss of PNPLA3 function may sensitize hepatocytes to other forms of hepatotoxicity. Therefore, the I148M variant causes progressive liver damage not through lipid-induced hepatocellular damage but rather through reduced detoxification capacity.

Our data, in large part, supports this hypothesis. We found that in addition to the global downregulation of metabolic pathways related to lipid metabolism, there was a similar downregulation in genes responsible for drug detoxification in PNPLA3-edited cells. Reduced expression of DMEs has the potential to cause accumulation of toxic compounds in hepatocytes and impair the ability of these cells to metabolize or excrete said compounds. This accumulation then leads to oxidative stress, mitochondrial dysfunction, and ultimately cell death. Patients with advanced NAFLD/NASH have reduced expression of DMEs and it is believed that this reduction is a contributing factor to the increased susceptibility to drug induced liver injury [262, 263, 267, 268]. Given this global downregulation of DMEs and the clinical evidence that this reduction contributes to liver injury, we compared the effect of *PNPLA3* genotype on the toxicity of three common hepatotoxins, alcohol, iron, and acetaminophen, in our system. We found that PNPLA3-edited cells were more susceptible to ethanol-induced toxicity; however, a similar trend was not present in the iron and acetaminophen toxicity assays. There are several potential explanations for this discrepancy. It is possible that the drug metabolism in our cells is not functionally mature enough to test this hypothesis reliably. The HLCs in our system are immature and phenotypically resemble foetal hepatocytes. Therefore, it is possible that differences in drug metabolism are not captured in our *in vitro* system due to this functional immaturity. Alternately, it is possible that iron and acetaminophen detoxification are not affected by *PNPLA3* genotype, but the metabolism of other drugs is affected. The increased toxicity of ethanol in PNPLA3-edited cells supports this hypothesis. We propose additional toxicity studies to fully elucidate the role of *PNPLA3* genotype in drug metabolism. For example, it would be prudent to examine the toxicity of the chemotherapeutic drugs that have been linked to increased toxicity in patients with

the I148M variant. Additionally, more mechanistic studies should be performed to understand how PNPLA3 genotype influences ethanol toxicity.

Data from our *in vitro* system indicates that loss of PNPLA3 function reduces the capacity of hepatocytes to metabolize drugs and other toxic metabolites. This reduced metabolic capacity increases the susceptibility of these cells to hepatotoxicity and drug induced liver injury. This increased hepatotoxicity does appear to be specific to certain drugs such as ethanol. Thus, we contend that carriers of I148M variant have a global downregulation of lipid metabolism that makes them less susceptible to lipotoxicity; however, these patients also have reduced drug metabolism capacity which makes them more susceptible to other forms of hepatotoxicity. Additional studies are needed to fully analyse the accuracy of this hypothesis. However, if this hypothesis is proven true, it could have major implications for the clinical management of patients. In addition to diet and exercise to decrease the NAFLD disease burden, the alcohol consumption and drug cocktail used to treat comorbidities would need to be carefully monitored.

7.4.2 PNPLA3 Causes NAFLD Without Insulin Resistance

Selective insulin resistance is a hallmark of NAFLD caused by metabolic disease. Insulin resistance in the liver causes uncontrolled de novo lipogenesis and hepatic glucose production. The increased concentration of glucose and free fatty acids derived from gluconeogenesis and de novo lipogenesis, respectively, leads to glucotoxicity and lipotoxicity in hepatocytes [64]. The metabolic dysregulation in insulin resistant hepatocytes leads to oxidative stress and mitochondrial dysfunction which causes inflammation through the activation of JNK and NF-κB. This inflammation then further exacerbates insulin resistance causing a vicious circle of liver damage inducing more liver damage [79-81]. However, despite being linked to increased disease severity, NAFLD patients carrying the PNPLA3 I148M variant can develop severe NAFLD in the absence of insulin resistance [138]. The mechanism by which these patients can progress toward severe disease without underlying insulin resistance remains a mystery; however, the lack of insulin resistance in I148M carriers indicates that the pathophysiology of PNPLA3-induced NAFLD is functionally distinct from obesity-induced NAFLD. Thus, it is not altogether unsurprising that PNPLA3-edited cells behave differently from genetically normal cells when presented with a lipid stressor.

A major effector of insulin signalling on lipid and glucose metabolism is the AKT signalling cascade. At high insulin levels, AKT is activated through phosphorylation. AKT phosphorylation then leads to upregulation of SREBP and downstream de novo lipogenesis genes as well as inhibition of gluconeogenesis via FOXO1 phosphorylation [21]. Since AKT is the major inhibitor of FOXO1 and downstream hepatic glucose production, disruption to this signalling cascade results in constitutively active gluconeogenesis [64]. However, SREBP is controlled by LXR as well as AKT so even in the absence of proper AKT signalling, insulin still maintains the ability to activate de novo lipogenesis [28]. Previous studies have shown that treatment with palmitic acid causes reduced insulin-mediated phosphorylation of AKT. However, by upregulating ChREBP or SCD1, cells increased the ratio of MUFAs to SFAs. This had the dual effect of increasing the incorporation of SFAs into triglycerides while restoring AKT signalling and insulin sensitivity. The enhanced insulin sensitivity was accompanied by decreased mitochondrial β-oxidation as well as reduced inflammation despite increased steatosis [29, 30]. Thus, insulin sensitivity can be maintained through increased incorporation of SFAs into triglycerides which restores AKT signalling and results in reduced β-oxidation, lipotoxicity, and inflammation. Therefore, we hypothesize that the reduced lipotoxicity seen in PNPLA3-edited cells treated with palmitic acid is the *in vitro* manifestation of intact insulin signalling.

The lipidomic data from PNPLA3-edited cells indicates that loss of PNPLA3 activity causes increased incorporation of SFAs into triglycerides as well as retention of PUFAs in triglycerides. Both of these observations align with clinical evidence showing that I148M carriers often maintain insulin sensitivity, have increased hepatic retention of PUFAs, and have reduced expression of SREBP1c and downstream de novo lipogenesis [138, 165, 241]. We believe that the incorporation of these two fatty acid moieties into triglycerides maintains insulin sensitivity in the HLCs. The increased incorporation of SFAs into triglycerides may prevent the deleterious effect of these fatty acids on insulin mediated AKT phosphorylation while the high concentration of PUFAs in the triglycerides of these cells leads to a downregulation of SREBP1c and downstream de novo lipogenesis genes. Therefore, it is possible that the reduced lipotoxicity of palmitic

acid in PNPLA3-edited cells is merely an epiphenomenon caused by the effort of these cells to maintain insulin sensitivity. This hypothesis is supported by the downregulation of metabolic as well as inflammatory pathways. By preventing insulin resistance, loss of PNPLA3 function dissociates steatosis from hepatocellular damage and inflammation. Additional studies are needed to understand the mechanism by which the I148M variant maintains insulin sensitivity.

7.4.3 PNPLA3 Differentially Affects Hepatocytes and Hepatic Stellate Cells to Manifest Different Aspects of the Disease

The intact insulin signalling in some PNPLA3 I148M carriers, however, is at odds with the increased NAFLD severity and higher necro-inflammatory scores of these patients. Inflammation and disease progression in NAFLD patients are usually inversely proportional to insulin sensitivity [56, 57, 276]. Given the clinical evidence as well as the data from our system, it appears that increased steatosis and increased hepatocellular damage are functionally dissociated in the livers of patients with the I148M variant in PNPLA3. The downregulation of metabolic pathways that generally lead to lipotoxicity as well as reduced inflammatory gene expression in PNPLA3-edited cells argue against a direct role of the I148M variant in causing hepatotoxicity, despite increased steatosis. However, the I148M variant has been linked to HSC activation as well as increased inflammation and fibrogenesis [113, 114, 132, 135]. Therefore, we hypothesized that the I148M variant affects hepatocytes and HSCs differently, causing each cell type to manifest a different aspect of the disease. We hypothesize that the I148M variant causes a downregulation of metabolism in hepatocytes which results in increased steatosis as well as reduced lipotoxicity. Meanwhile, the I148M variant causes activation of HSCs which are responsible for the inflammation, fibrosis, and general disease progression.

The data from our *in vitro* system is consistent with this hypothesis. We found that loss of PNPLA3 function, either through the I148M variant or a complete PNPLA3 protein knock-out, led to increased steatosis. The increased lipid accumulation that characterized the PNPLA3-edited cells was caused by concomitant upregulation of triglyceride synthesis and downregulation of lipid catabolic pathways. The increased steatosis in these cells did not appear to harm the HLCs in any way. In fact, the increased steatosis appeared to protect the HLCs from ER stress and lipotoxicity caused by palmitic acid. These

findings support the growing body of evidence showing that steatosis is not necessarily cytotoxic and likely represents an epiphenomenon in NAFLD pathogenesis [35, 117, 279, 280]. In addition to the findings of our system, clinical evidence also supports this hypothesis. Clinical studies have correlated the I148M variant in PNPLA3 to steatosis, inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma in numerous aetiologies of liver disease. The variant is less often correlated with markers of hepatocyte damage such as hepatocellular ballooning and the presence of Mallory bodies [132, 151]. These clinical data indicate that the liver damage in carriers of the I148M variant may be independent of hepatocyte damage.

The I48M variant in PNPLA3 confers increased risk for fibrosis independent of other markers of disease severity [132, 135]. Therefore, we contend that HSCs are responsible for liver damage in carriers of the I148M variant independent of steatosis in hepatocytes. PNPLA3 participates in HSC activation by promoting the extracellular release of retinol from HSCs, the first step in activation [112]. HSCs with the I148M variant retain less retinol than their I148I counterparts which causes higher expression of profibrotic genes [114]. Additionally, HSCs expressing the I148M variant have higher proliferation rate and chemotaxis, both hallmarks of activated HSCs. HSCs with the I148M variant also secrete proinflammatory markers such as CCL2, CCL5, and TGFβ which causes increased recruitment of inflammatory cells like macrophages [113]. Given these data, it is reasonable to hypothesize that HSCs control the inflammatory and fibrotic response in PNPLA3-induced NAFLD. This hypothesis offers insight into how the I148M variant increases the risk of severe liver damage in several different aetiologies of liver disease despite divergent pathogenesis. By conferring a proinflammatory and profibrotic phenotype to HSCs, the I148M variant contributes to progressive liver damage independent of other disease processes.

We believe that this hypothesis offers the most reasonable explanation for the role of the I148M variant in chronic liver diseases such as NAFLD given the data from our system and the existing clinical and experimental evidence. Patients with the I148M variant have increased hepatic steatosis and the triglycerides stored in the livers of these patients are enriched for PUFAs. The high concentration of PUFAs as well as the insulin sensitivity

in the livers of these patients are cytoprotective from lipid-induced stress, as confirmed in our *in vitro* system. Though the global downregulation of lipid metabolism that results in triglyceride accumulation may be cytoprotective against lipotoxicity, it is accompanied by a concomitant downregulation of xenobiotic metabolism which may reduce the detoxification capacity of hepatocytes and result in increased sensitivity to other hepatotoxins such as ethanol. Additionally, the predilection of the HSCs in carriers of the I148M variant toward a proinflammatory, profibrotic phenotype causes inflammation, hepatocellular damage, and progressive fibrosis in these patients. It is possible that the steatosis in I148M carriers is an epiphenomenon or that the increased steatosis in the hepatocytes sensitizes these cells to hepatocellular damage mediated by activated HSCs. In order to fully elucidate the pathogenic mechanism of the PNPLA3 I148M variant, the cell to cell interactions between hepatocytes and HSCs carrying the risk allele must be fully characterized.

7.5 Limitations of Our Model

Our system is the first of its kind to model PNPLA3-associated NAFLD. By utilizing CRISPR/CAS9 and hiPSCs, we were able to create an *in vitro* system to analyse the effect of PNPLA3 genotype on HLCs and their response to lipid induced stress. Our system is the first to model the I148M variant at endogenous levels in a fully human system. However, despite the numerous unique advantages, our system still has quite a few limitations.

First, the differentiated HLCs are not fully mature and more closely resemble foetal hepatocytes than adult hepatocytes. Therefore, it is possible that the metabolic phenotype of these cells may not be fully representative of the adult phenotype or disease. However, the HLCs in our system did respond to lipid supplementation in a predictable manner. The PNPLA3^{UC} cells became steatotic when treated with the MUFA oleic acid and suffered from lipotoxicity when treated with the SFA palmitic acid. Thus, we are reasonably confident that the HLCs in our system have a functional lipid metabolism. Additionally, the HLCs were susceptible to drug induced toxicity when treated with both ethanol and acetaminophen indicating that the drug metabolism of these cells was intact. So even though these cells are not fully functionally mature, they are metabolically active

and represent a reasonable model system to study the role of PNPLA3 in NAFLD progression.

Second, the *in vitro* nature of this system lends a certain degree of artificiality to the model. In our system we used two different fatty acid species to model different aspects of NAFLD. However, *in vivo*, hepatocytes would not encounter a single moiety of fatty acid. Instead, hepatocytes would be subjected to a complex mixture of several different fatty acids belonging to each of the different fatty acid classes, SFAs, MUFAs, and PUFAs. It is possible that this complex, dynamic nutritional environment would alter the phenotype of PNPLA3-edited cells. Additionally, we examined only the effect of fatty acid supplementation in our system. Other nutritional factors such as fructose have been shown to significantly affect NAFLD development and progression [55, 63, 81]. In order to confirm the phenotype of PNPLA3-edited cells, additional studies are needed to examine the effects of complex nutritional signalling on steatosis and cell stress pathways in these cells. Beyond the lack of nutritional diversity, the HLCs in our *in vitro* system are not exposed to the complex network of cytokines and adipokines that influence the metabolic behaviour of hepatocytes *in vivo*. NAFLD is a multi-system disease that involves significant cross talk between several metabolic organs including the liver, adipose tissue, the gut, and the pancreas [56, 151]. Hepatocytes in the proper signalling environment may exhibit an entirely different phenotype that we are unable to capture in our *in vitro* system. However, the relative simplicity of this system does allow us to dissect the role of the I148M variant in hepatocytes without introducing confounding factors that may mask the mechanism of action. In this way, the simplicity may offer unique advantages as well.

Third, this thesis only examined the effect of *PNPLA3* genotype on the phenotype of hepatocytes. NAFLD is a complex metabolic disease that involves intercellular signalling between several hepatic cell types. PNPLA3 is known to be expressed in several cell types that are relevant to NAFLD including hepatocytes, stellate cells, and macrophages. Our system offers the opportunity to dissect the role of PNPLA3 pathogenesis on hepatocytes from the other hepatic cells in which it is expressed. As the main metabolic cell of the liver, hepatocytes are the site of steatosis which is a hallmark of NAFLD. In this work,

we demonstrated that loss of PNPLA3 function is a direct cause of the increased steatosis found in the livers of I148M carriers. However, we also demonstrated that loss of PNPLA3 is protective against lipotoxicity which is counterintuitive to the data linking I148M to more severe disease phenotypes, though this phenomenon may be unique to our *in vitro* challenge model and not fully reflective of *in vivo* response. It is possible that unique signals are produced by hepatocytes carrying the I148M variant that leads to increased recruitment or activation of macrophages and stellate cells. This nuance would not be captured in our model which has only a single cell type. Alternately, the I148M variant could uniquely influence the phenotypes of other hepatic cell types toward a proinflammatory or profibrotic state leading to increased liver damage and disease progression. In order to fully understand the pathogenesis of this disease and the contribution of PNPLA3, additional studies are needed. Recent advances in 3D organoid culture with many cell types may provide additional opportunities to further elucidate the mechanism of PNPLA3 in NAFLD.