8 Future Directions and Conclusions

8.1 Future Directions

In this thesis, we developed an *in vitro* culture system using CRISPR/CAS9 and hiPSCs to study the effect of *PNPLA3* genotype on NAFLD development in hepatocytes. We were able to replicate several key clinical findings including increased steatosis in PNPLA3-edited cells as well as the unique lipidomic signature of hepatic retention of PUFAs in triglycerides. Additionally, we confirmed for the first time in a fully human model system that the I148M variant is a loss of function variant that causes steatosis by downregulating metabolic pathways likely through reduced lipid droplet remodelling capabilities. The findings from this thesis also raised several questions about (1) how loss of PNPLA3 function leads to the global downregulation of lipid metabolism, (2) why loss of PNPLA3 function appears to be cytoprotective against lipotoxicity in hepatocytes, (3) how increased steatosis in PNPLA3-edited cells may lead to the increased liver injury that characterizes PNPLA3-induced NAFLD, (4) what role the PNPLA3 I148M variant plays in the phenotype of other hepatic cell types, and (5) how these other cell types may contribute to the pathogenesis of NAFLD. The work described in this thesis provides a first step in understanding the pathogenesis of PNPLA3-induced NAFLD; however, additional studies are needed to examine the mechanism by which the I148M variant in PNPLA3 causes increased steatosis and liver damage to induce NAFLD development and progression.

8.1.1 In-Depth Analysis of Signalling Pathways Influenced by PNPLA3

The RNA sequencing of the three genotypes of HLCs revealed an interesting pattern of downregulated genes involved in lipid homeostasis. This analysis in addition to the increased steatosis and reduced lipotoxicity in PNPLA3-edited cells suggests that loss of PNPLA3 function results in downregulation of lipid catabolic genes in favour of triglyceride formation. However, additional studies are needed to identify that signalling cascades that are directly affected by loss of PNPLA3. Very little is known about the role of PNPLA3 in the larger network of lipid metabolism and this *in vitro* system could be used to analyse the downstream genes that are influenced by *PNPLA3* genotype. Using the $PNPLA3^{UC}$ and $PNPLA3^{KO}$ lines, it may be possible to parse which signalling networks PNPLA3 participates in and which genes are directly affected by PNPLA3 functionality.

In addition, this system can be used to understand how carriers of the PNPLA3 I148M variant may be able to maintain insulin sensitivity in the context of obesogenic stimuli. It is possible that the inability of PNPLA3-edited cells to differentiate between saturated and unsaturated fatty acids are involved in this process. SFAs and the downstream cell stress responses have been directly implicated in the development and exacerbation of insulin resistance [81, 281]. In order to explore this possibility, we would need to first confirm that the insulin signalling pathway is intact and functional in our HLCs. Following such confirmation, insulin sensitivity assays could be used to analyse how PNPLA3^{UC} and PNPLA3-edited cells are differentially affected by oleic acid and palmitic acid supplementation. In this way, the *in vitro* system could easily be used to test the hypothesis that escape from palmitic acid-induced lipotoxicity is the *in vitro* manifestation of insulin sensitivity.

The relative simplicity of this *in vitro* system would be a major asset in the mechanistic analysis of the role of PNPLA3 in lipid metabolism and insulin signalling. Since the I148M variant has been proven to be a loss of function variant in our system, the PNPLA3^{KO} cells can be used to cleanly dissect the role of PNPLA3 in hepatocytes. Additionally, the supplementation of a single fatty acid to the *in vitro* system will allow us to understand with fine granularity how the saturation status of a lipid affects PNPLA3 and its downstream targets.

8.1.2 Proteomic Analysis of Lipid Droplets

We hypothesized based upon our lipidomics data and the available clinical data that the I148M variant causes a loss of lipid droplet remodelling function. Specifically, the loss of PNPLA3 function prevents the transfer of PUFAs from triglycerides to phospholipids causing a hepatic retention of these fatty acids. We hypothesized that this lack of lipid droplet remodelling affected the access of other lipid droplet proteins to the fatty acids contained in lipid droplets. Thus, causing a reduced flux of lipid precursors to metabolic pathways and resulting in the downregulation of the affected pathways. In order to test this hypothesis, we would need to perform proteomic analysis on the lipid droplets of PNPLA3^{UC} and PNPLA3-edited cells. This proteomic analysis would allow us to

determine if the reduced lipid droplet remodelling capacity of PNPLA3-edited cells alters the protein coating of lipid droplets. Further analyses of the differences in lipid droplet proteins would allow us to determine if loss of PNPLA3 reduces metabolic processes through an indirect mechanism by reducing lipid transport out of lipid droplets or through a more direct mechanism.

8.1.3 Long-Term Culture to Model Chronic Nature of NAFLD

Our analyses have demonstrated that the loss of PNPLA3 function has a profound effect on the transcriptomic and lipidomic profiles of HLCs. Loss of PNPLA3 function causes global downregulation of metabolic pathways and improper responses to lipid-induced stress. We hypothesize that this metabolic dysregulation will have a negative impact on the long-term-health of the cell. Since NAFLD is a chronic disease that takes decades to develop and progress toward end-stage liver disease, it is likely that our model, which has the longevity of only one week, did not capture the long-term effects of metabolic dysregulation. Additional studies are needed to examine how the global downregulation of lipid metabolism affects ER stress and inflammatory pathways over time. In order to accomplish these studies, the *in vitro* system would need to be adjusted to allow for longer term culture. Once the longevity of the culture can be extended, time-course analyses should be undertaken to analyse the effect of *PNPLA3* genotype on viability, lipotoxicity, and inflammation of palmitic acid treated cells to understand how metabolic dysregulation may contribute to long-term hepatocellular damage and disease progression.

8.1.4 Additional Drug Toxicity Assays

Our preliminary results indicate that loss of PNPLA3 function may increase the susceptibility of hepatocytes to drug-induced cytotoxicity. Clinical evidence supports this hypothesis as carriers of the I148M variant are more susceptible to liver damage induced by ethanol and chemotherapeutic drugs. In our system, we found that PNPLA3-edited cells downregulated the expression of DMEs and were more susceptible to ethanol-induced toxicity. However, the PNPLA3-edited cells were not more susceptible to toxicity induced by iron overload or acetaminophen overdose. Thus, additional studies are needed in order to confirm or refute this hypothesis as a potential mechanism for increased liver injury in patients with the I148M variant.

It would be important to confirm the increased toxicity of ethanol in PNPLA3-edited cells as well as explore the mechanism by which ethanol causes toxicity in the context of PNPLA3 loss of function. Ethanol is consumed at non-harmful doses by a large portion of the population but if the results in this thesis are confirmed, even small doses of ethanol could be extremely deleterious to patients carrying the I148M variant. Thus, this finding could have significant clinical implications for the management of NAFLD patients with the I148M variant. In addition, we propose testing the toxicity of several other drugs that have the potential to cause hepatocellular damage in patients with the I148M variant. The toxicity of drugs that have been connected to hepatotoxicity in I148M carriers clinically as well as drugs that are specifically metabolized by phase one DMEs that were specifically downregulated in the PNPLA3-edited HLCs should be assessed.

8.1.5 Co-Culture Model for a More Physiologically Relevant Model of NAFLD

NAFLD induced by the I148M variant in PNPLA3 has implicated several cell types in the pathogenesis of the disease. Hepatocytes are the main metabolic hub in the liver and the site of steatosis. HSCs are the hepatic cell type responsible for fibrosis and these cells also play an important role in coordinating the inflammatory response in NAFLD. Finally, macrophages coordinate with immune cells and HSCs to control the proinflammatory environment that causes disease progression and characterizes NASH. The two most commonly associated hepatic cell types with PNPLA3-induced NAFLD are hepatocytes and HSCs; however, preliminary studies in our lab suggest that PNPLA3 is also expressed in macrophages and thus could influence the inflammatory aspect of the disease (data not shown). The interplay of each of these cell types is important in the pathogenesis of NAFLD and therefore, all three of these cell types would be necessary to properly model NAFLD progression *in vitro*. We propose expanding on the findings in this thesis by creating a more physiologically relevant co-culture model of PNPLA3-induced NAFLD. The three genotypes of hiPSCs could be differentiated into hepatocytes, to model the metabolic aspects of NAFLD, HSCs, to model fibrosis, and macrophages, to model inflammation before combining the three hepatic cell types together into a single co-

culture system to model PNPLA3-induced NAFLD *in vitro*. A schematic of the proposed co-culture model is shown in Figure 8.1.

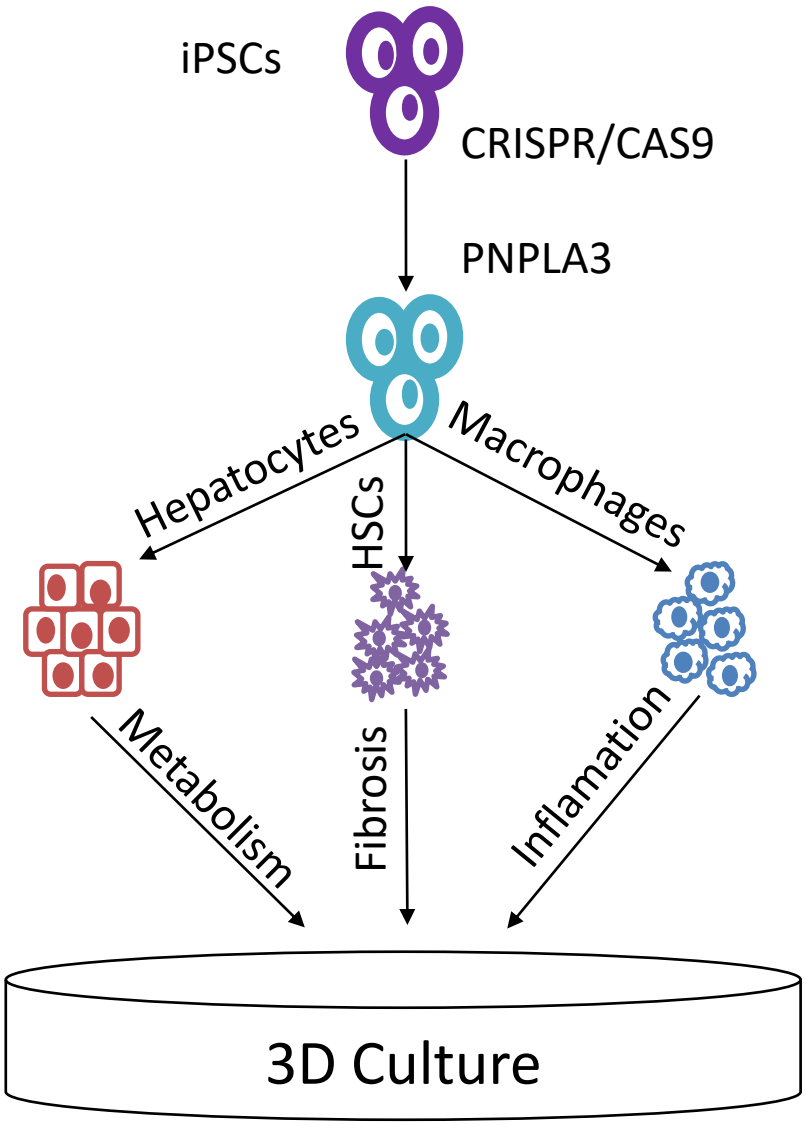

Figure 8.1 Co-Culture Model Schematic.

Normal hiPSCs were edited using CRISPR/CAS9 in order to generate PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} hiPSC lines. These lines can then be differentiated into the three hepatic cell types that are most relevant for NAFLD development and progression: hepatocytes to represent the metabolic aspects of the disease, HSCs to model fibrosis, and macrophages to model inflammation. Following differentiation, the three cell types can be combined into a 3D co-culture to create an *in vitro* physiological model of PNPLA3-induced NAFLD.

Well-characterized differentiation protocols exist for each of the proposed hepatic cell types making the creation of this model readily feasible [184, 194, 282]. This system would capture the complex cellular interactions and intercellular signalling that cause progression from simple steatosis through NASH and fibrosis. In addition, since each of the cell types would be differentiated from the same hiPSC line, the system would be derived from a single genetic background which eliminates potential confounding variables from the model. This co-culture system shows the true advantage of using hiPSCs and CRISPR/CAS9 to study the effects of *PNPLA3* genotype on liver disease as multiple hepatic cell types can be derived from a single line. This physiologically relevant 3D co-culture model would be the first of its kind that simulates the effect of genotype on a complex chronic liver disease. A model of this type would largely reduce the need to use the less appropriate murine model to understand the function of PNPLA3 and the contribution of the I148M variant to NAFLD development and progression.

8.1.6 CRISPRi Screen for Mechanistic Insight

As extensively discussed throughout this thesis, the function of PNPLA3 in healthy tissues and NAFLD pathogenesis is subject to great debate in the scientific community. Over a decade of research has failed to fully characterize this protein despite its huge impact on public health. We propose the use of our *in vitro* system as well as the CRISPRi screening technology to help elucidate the mechanism by which PNPLA3 influences metabolism as well as identify potential druggable targets to reduce NAFLD risk in patients carrying the I148M variant. A CRISPRi screen performed on PNPLA3^{UC} cells would allow us to identify genes that contribute to the increased steatosis and reduced lipotoxicity that characterize PNPLA3-edited cells. The PNPLA3^{I148M} and PNPLA3^{KO} cells could be used as a unique internal control to dissect incidental findings from genes truly associated with PNPLA3. The CRISPRi system offers the opportunity to quickly and easily analyse which metabolic pathways PNPLA3 may play a role in as well as downstream genes that are repressed by loss of PNPLA3 function which are responsible for the phenotypic behaviour of PNPLA3-edited cells. Alternately, the CRISPRi screen could be used on PNPLA3^{KO} cells to identify potential drug targets that improve the disease phenotype. This CRISPRi screen would offer novel insight into treatment options that are unique to carriers of the I148M variant in PNPLA3.

8.1.7 Humanized Mouse Model

As discussed, *in vitro* disease models have an inherent amount of artificiality due to the difficulty in modelling the complex, multi-system crosstalk that happens *in vivo*. In order to overcome this challenge, we propose the development of humanized mouse models using cells derived from the PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} hiPSC lines. As described for the proposed *in vitro* co-culture model, hiPSCs from each of the three genotypes could be differentiated into relevant hepatic cell types before being injected into the livers of humanized mice for engraftment [283, 284]. Following engraftment, the mice could be fed with a high fat diet to cause a NAFLD phenotype before being sacrificed and having their livers collected for histology. This humanized mouse model would take advantage of the physiological *in vivo* environment without the confounding factor of differential PNPLA3 expression and functionality between humans and mice. This model would allow us to study the effect of organ to organ crosstalk on the development and progression of NAFLD for each of the *PNPLA3* genotypes. Additionally, the humanized mouse model represents the ideal model system to test the long-term effects of PNPLA3-induced metabolic dysregulation on NAFLD phenotype because mice can live for several months as opposed to weeks as with *in vitro* models. This humanized mouse model would be a massive contribution to the field and would represent the most physiologically relevant model of human PNPLA3-induced NAFLD to date.

8.2 Conclusions

According to the University of Cambridge guidelines for the award of a PhD Degree, the candidate must demonstrate that his or her dissertation "represents a significant contribution to learning, for example through the discovery of new knowledge, the connection of previously unrelated facts, the development of new theory, or the revision of older views". To this end, this dissertation has accomplished such criteria through the following

- Developing an *in vitro* human model to elucidate the role of PNPLA3 in NAFLD development and progression
- Conclusively demonstrating that the I148M variant in PNPLA3 is a loss of function variant that causes steatosis in hepatocytes
- Discovering that cells lacking PNPLA3 functionality are resistant to lipotoxicity induced by SFAs due to increased incorporation of these fatty acids into triglycerides
- Confirming the role of PNPLA3 in remodelling phospholipids in lipid droplets in a PUFA direction
- Demonstrating that loss of PNPLA3 function results in a global downregulation of genes involved in the metabolism of lipids and drugs
- Discovering that loss of PNPLA3 function causes increased susceptibility to hepatotoxins such as ethanol

Collectively, the results presented in this thesis have advanced both the knowledge and tools available to the field and will significantly contribute to several exciting future developments in the years to come.