6 MECHANISTIC INSIGHTS INTO HOW THE I148M VARIANT IN PNPLA3 CONTRIBUTES TO NAFLD PATHOGENESIS

6.1 Introduction

In our investigations thus far, we have found evidence that reduced PNPLA3 enzymatic activity leads to a reduction in lipid catabolism in favour of lipid anabolism. Loss of PNPLA3 function either through the I148M variant or knock-out of the PNPLA3 gene leads to increased lipid accumulation in the form of neutral triglycerides. This increased lipid accumulation appears to be cytoprotective against SFAs. Based upon the lipidomics data in the previous chapter, we showed that PNPLA3^{I148M} and PNPLA3^{KO} cells are able to escape palmitic acid-induced lipotoxicity by preferentially incorporating the SFAs into triglycerides rather than other metabolic processes such as oxidation. Accordingly, the PNPLA3-edited cells appear to process SFAs in a manner more similar to MUFAs such as oleic acid, causing a steatotic rather than a lipotoxic phenotype.

The lipidomics data further demonstrated a downregulation in lipid droplet remodelling which caused the accumulation of PUFAs in the triglycerides of PNPLA3-edited cells. The reduced lipid droplet remodelling capacity of these cells could have several, major implications for overall lipid metabolism. The content of the lipid droplets as well as the lipid composition of the organelle's membrane both play a role in determining the protein composition of the lipid droplet membrane [248, 256]. The failure of PNPLA3-edited cells to remodel the triglyceride and phospholipid pools in lipid droplets may result in an altered protein composition on the lipid droplet membranes in these cells. This altered protein composition may interfere with lipid homeostasis as well as intracellular signalling pathways. PNPLA3-edited cells sequester large quantities of fatty acids and other lipid species in their lipid droplets resulting in less availability of fatty acids for other metabolic processes. Additionally, this sequestration interferes with intracellular lipid signalling processes. Lipids are the major endogenous ligands that activate or repress transcription factors involved in metabolism and other cellular functions. Sequestration of lipids in lipid droplets may prevent activation of lipid catabolic pathways by failing to activate PPARα and downstream oxidation processes [257, 258]. Failure to properly regulate lipid metabolism may result in short term benefits such as escape from SFA-induce lipotoxicity; however, this dysregulated metabolism may have long term negative impacts on the health of the hepatocyte. Long-term dysregulation of lipid metabolism has

the potential to contribute to the inflammatory state as well as negatively impact other metabolic pathways such as drug metabolism or detoxification mechanisms in these cells.

In addition to maintaining lipid homeostasis in the body, the liver plays an important role in drug detoxification. It is estimated that the liver is responsible for clearing up to 60% of prescribed drugs. The purpose of drug metabolism is to facilitate the elimination of endogenous and exogenous molecules from the body by converting lipophilic compounds to hydrophilic products. This metabolism can be divided into four phases. Phase zero enzymes, usually belonging to the solute carrier (SLC) transporter family, are uptake transporters that facilitate the import of drugs into the hepatocyte. Phase one enzymes modify the drugs to become more water soluble, most commonly by oxygenating the drug. Enzymes that fall into the phase one category include cytochrome P450 (CYP) enzymes as well as several classes of dehydrogenases. The expression of these phase one enzymes is under the control of several transcription factors including CAR and PXR and downregulation of these enzymes generally leads to increased hepatotoxicity. The phase two enzymes are responsible for conjugating phase one products to hydrophilic compounds. Glucuronidation, facilitated by uridine diphospho-glucuronosytransferases (UGTs), sulfation, carried out by sulfotransferases (SULTs), and glutathionylation, performed by glutathione s-transferases (GSTs), are the most common conjugation reactions. Following detoxification, the hydrophilic products are exported by phase three enzymes. These efflux transporters are generally members of the ATP binding cassette (ABC) transporter family also known as the multidrug resistant proteins [50, 259-261].

There is significant overlap between lipid metabolism and drug metabolism in hepatocytes. These two metabolic processes share many of the same transporters, detoxifying enzymes, and transcriptional regulators. Both lipids and xenobiotic compounds can be imported and exported from hepatocytes using the ABC and SLC transporters. Since both lipids and most xenobiotics are hydrophobic in their un-metabolized state, it is efficient for hepatocytes to use the same transporters for both classes of molecules. The most prolific class of phase I drug metabolizing enzymes (DMEs) is the CYP family of enzymes. Humans express over 50 different CYP isoforms with different substrate specificity and functionality. CYPs are responsible for

metabolizing up to 75% of all prescribed xenobiotics as well as endogenous lipids such as PUFAs, retinol, bile acids, and steroids [51]. Thus, CYP enzymes are responsible for metabolizing both exogenous drugs and endogenous lipid species.

Given the overlap in the enzymes used to metabolize both drugs and lipids, there should be some overlap in the transcriptional regulation of these two pathways. Several transcription factors have been implicated in activation of both lipid and drug metabolism pathways. Notably, HNF4α is the master regulator of hepatocyte function and plays a key role in regulating gene expression of genes involved in both drug metabolism and lipid homeostasis. HNF4α activity is modulated by fatty acids and this transcription factor has significant crosstalk with other nuclear receptors active in lipid and drug metabolism including PPARα, PXR, and CAR. PPARα has been shown to activate both lipid β-oxidation pathways as well as upregulate the expression of CYP3A4 which is responsible for metabolizing up to 50% of commercially available drugs [50, 259, 262]. PPARα has also been implicated in modulating the expression of PXR and CAR, two major transcription factors responsible for activating DMEs. In addition to its role in drug metabolism, PXR also plays a role in controlling triglyceride homeostasis.

The crosstalk between lipid and drug metabolism pathways is further exemplified by the influence of NAFLD on drug metabolism. Patients with NAFLD are more susceptible to severe drug induced liver injury and hepatotoxicity. This hepatotoxicity is due to a combination of increased oxidative stress and reduced expression of DMEs. Excess fat accumulation in the liver results in a global downregulation of all phases of drug metabolism [262-265]. In order to protect hepatocytes from accumulation of toxic drugs, the phase zero transporters are downregulated to decrease drug uptake into hepatocytes. Once drugs enter the hepatocyte, both phase one and two detoxifying enzymes tend to be downregulated in NAFLD. NASH causes the downregulation of nearly all drug metabolizing CYP enzymes including CYP3A4, CYP2C9, CYP2A6, CYP2B6, and CYP2D6. The exception to this trend is CYP2E1 which is consistently elevated in the livers of NAFLD patients [264, 266, 267]. The upregulation of CYP2E1 leads to the exhaustion of glutathione reserves which causes oxidative stress and liver damage in these patients. Similarly, phase two DMEs are largely downregulated as well. UGT2B7,

SULT1A2, and GST activity were all downregulated in the livers of NASH patients [263, 268]. Together these data indicate that dysregulation of lipid metabolism, as in NASH, causes a reduction in the drug metabolism capacity of hepatocytes. This reduced drug metabolism leads to increased risk of hepatotoxicity and drug-induced liver injury.

Due to its role in lipid metabolism and the pathogenic role for the I148M variant in lipid accumulation, it is possible that PNPLA3 may also be implicated in drug metabolism. Indeed, PNPLA3 has been clinically correlated with several liver diseases in which detoxification of endogenous and exogenous compounds plays a key role. PNPLA3 has been strongly correlated with worse prognosis in patients with ALD, hereditary hemochromatosis, and cancer patients undergoing chemotherapy [139, 143, 224, 225]. The I148M variant has been linked to elevated serum levels of aminotransferases, increased steatosis, fibrosis progression, and cirrhosis development in these patients. These clinical data offer strong evidence that PNPLA3 may play a role in drug metabolism as well as lipid metabolism.

In this chapter, we sought to explore the mechanism by which the PNPLA3 I148M variant interferes with lipid and drug metabolism. We began by analysing the transcriptomic data collected from the PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells treated with and without fatty acid supplementation. We used this transcriptomic data to understand the pathways that were perturbed by changes in the PNPLA3 genotype with a particular focus on lipid and drug metabolism pathways. Following this analysis, we used our *in vitro* model system to validate these findings and test the hypothesis that loss of PNPLA3 function may have a deleterious effect on drug metabolism. In order to test this hypothesis, we examined the effect of ethanol, iron, and acetaminophen toxicity on the viability of PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells.

6.2 PNPLA3 edited cells downregulate metabolic pathways following lipid exposure

As demonstrated in both patient cohorts and our *in vitro* system, alterations to PNPLA3 result in stark phenotypic and lipidomic differences. These differences are likely due to fundamental alterations to existing metabolic processes; however, over a decade of

research dedicated to PNPLA3 has failed to uncover the mechanism of pathogenesis of the I148M variant in NAFLD. We believe that our system offers a unique opportunity to understand the transcriptomic differences between the three genotypes as well as gain insight into how the different genotypes respond to lipid induced stress. Using the RNA sequencing data described in Chapter 4 of this thesis, we sought to understand how changes to the PNPLA3 genotype altered gene expression and cellular pathways in an effort to gain mechanistic insight into the pathology of the I148M variant. As a reminder, the RNA sequencing was performed on PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells that had been differentiated into HLCs, placed into 3D culture, and treated for 24 hours with either control, oleic acid, or palmitic acid supplemented medium. Following sequencing, we performed principle component analysis, differential gene expression analysis, and gene ontology analysis to examine the differential transcriptomic response of the three genotypes to lipid induced stress. Given the phenotypic differences that we previously observed, we hypothesized that PNPLA3-edited cells would have similar transcriptomic profiles regardless of lipid treatment and that these cells would downregulate pathways involved in lipid catabolism and cell stress.

We began by performing a gene ontology enrichment analysis on the top 500 differentially expressed genes in order to identify pathways that are up or downregulated in PNPLA3-edited cells compare to untargeted control. We found in Chapter 4 that PNPLA3^{KO} cells exhibited the most extreme phenotype and displayed the most profound differences from PNPLA3^{UC} cells. Given that PNPLA3^{I148M} cells tended to be intermediate between PNPLA3^{UC} and PNPLA3^{KO} cells in all analyses, we chose to perform our gene ontology analyses using solely PNPLA3^{UC} and PNPLA3^{KO} cells in order to maximize the effect size. Since we have proven in our system that the I148M variant is a loss of function variant and exhibits an intermediate phenotype between untargeted control and knock-out genotypes, we believe that the results of these analyses can be used to infer how loss of PNPLA3 function via the I148M variant would affect hepatocyte function.

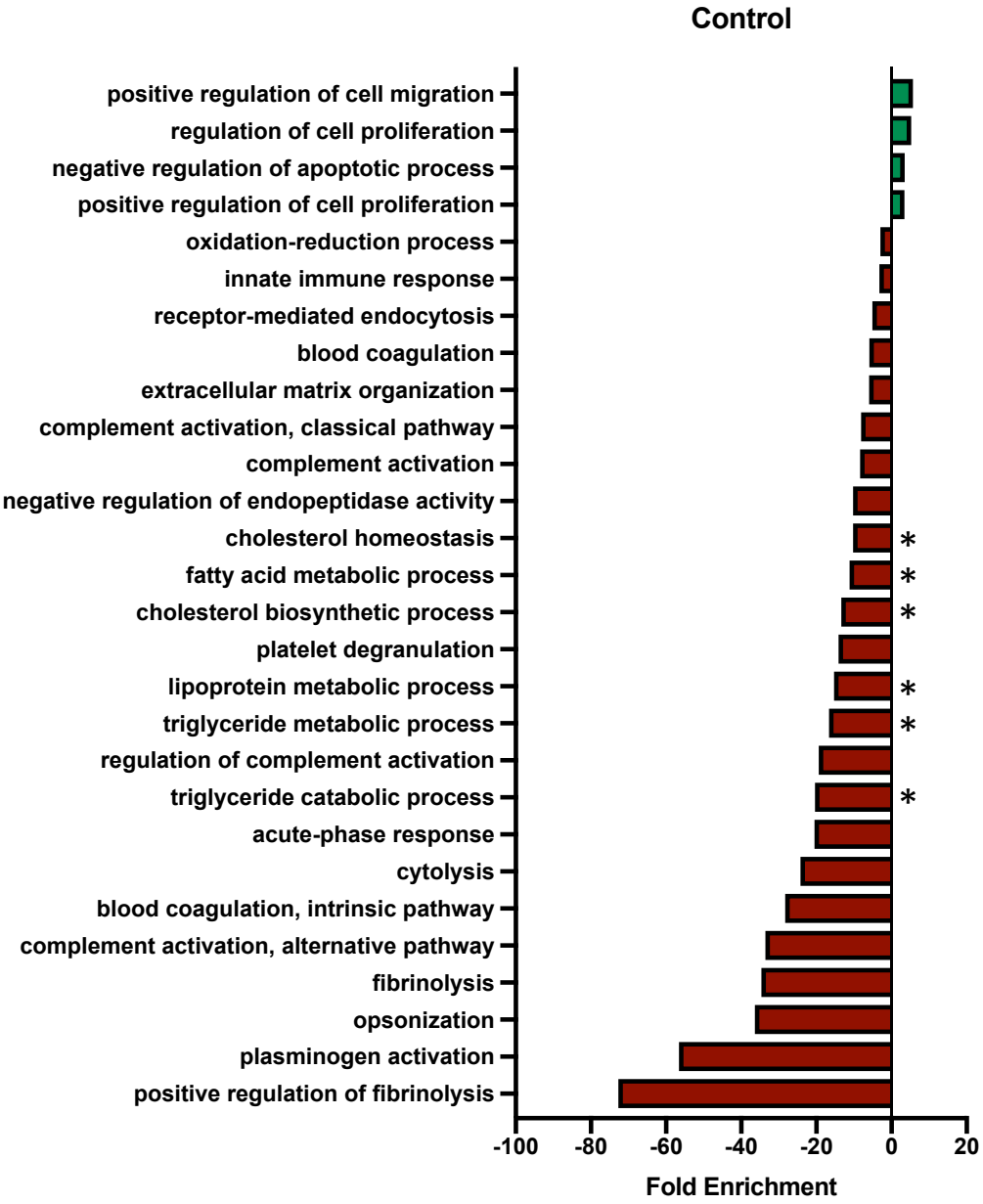

Figure 6.1 Gene ontology enrichment analysis for PNPLA3^{KO} and PNPLA3^{UC} cells treated with control medium.

Gene ontology enrichment analysis comparing PNPLA3^{UC} and PNPLA3^{KO} cells treated with control medium. Statistically significantly upregulated pathways are shown in green and downregulated pathways are shown in red. Metabolic processes are marked with a star. These results indicate that PNPLA3^{KO} cells downregulate several metabolic pathways compared to PNPLA3^{UC} cells regardless of treatment type.

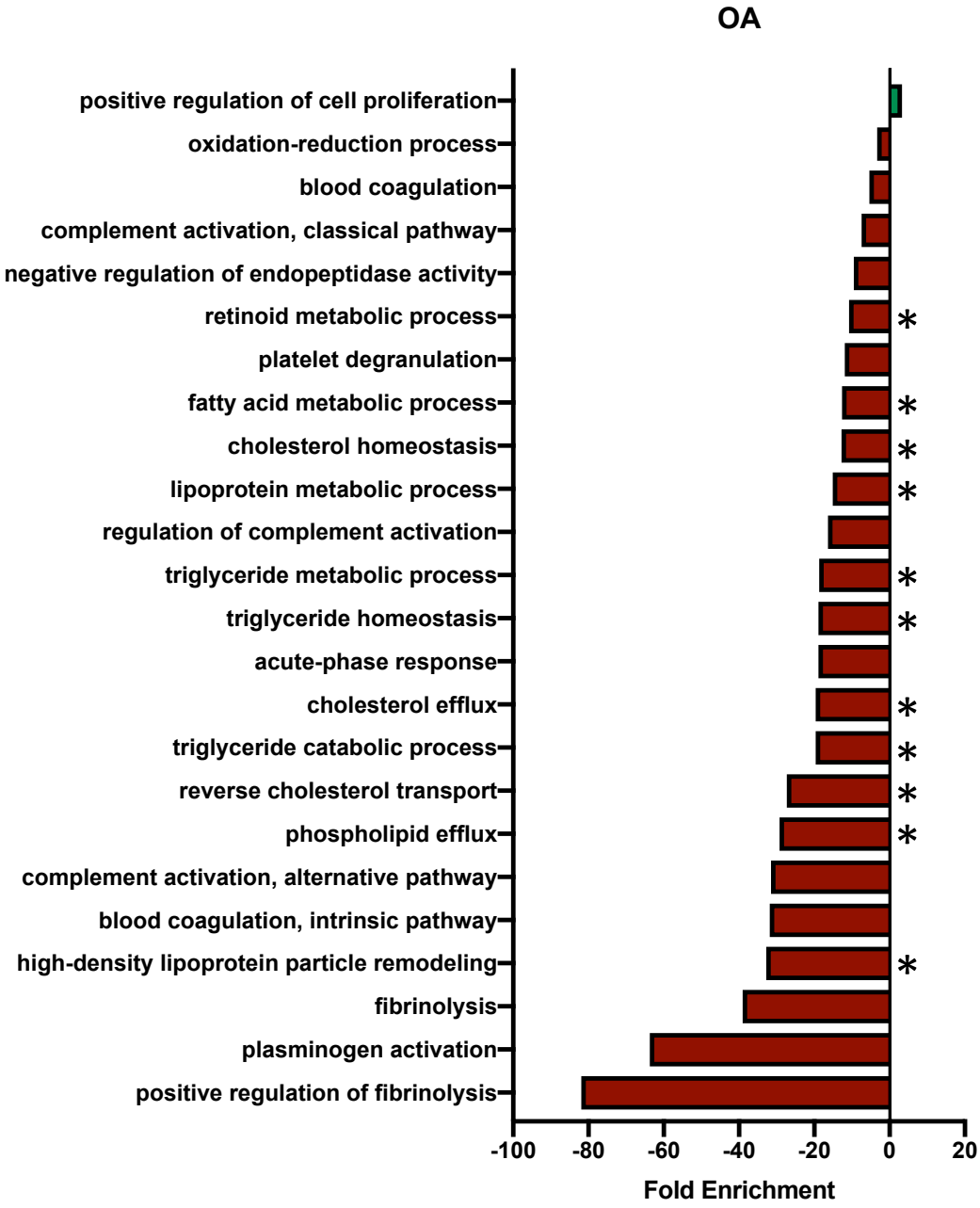

Figure 6.2 Gene ontology enrichment analysis for PNPLA3^{KO} and PNPLA3^{UC} cells treated with oleic acid.

Gene ontology enrichment analysis comparing PNPLA3^{UC} and PNPLA3^{KO} cells treated with oleic acid. Statistically significantly upregulated pathways are shown in green and downregulated pathways are shown in red. Metabolic processes are marked with a star. These results indicate that PNPLA3^{KO} cells downregulate several metabolic pathways compared to PNPLA3^{UC} cells regardless of treatment type.

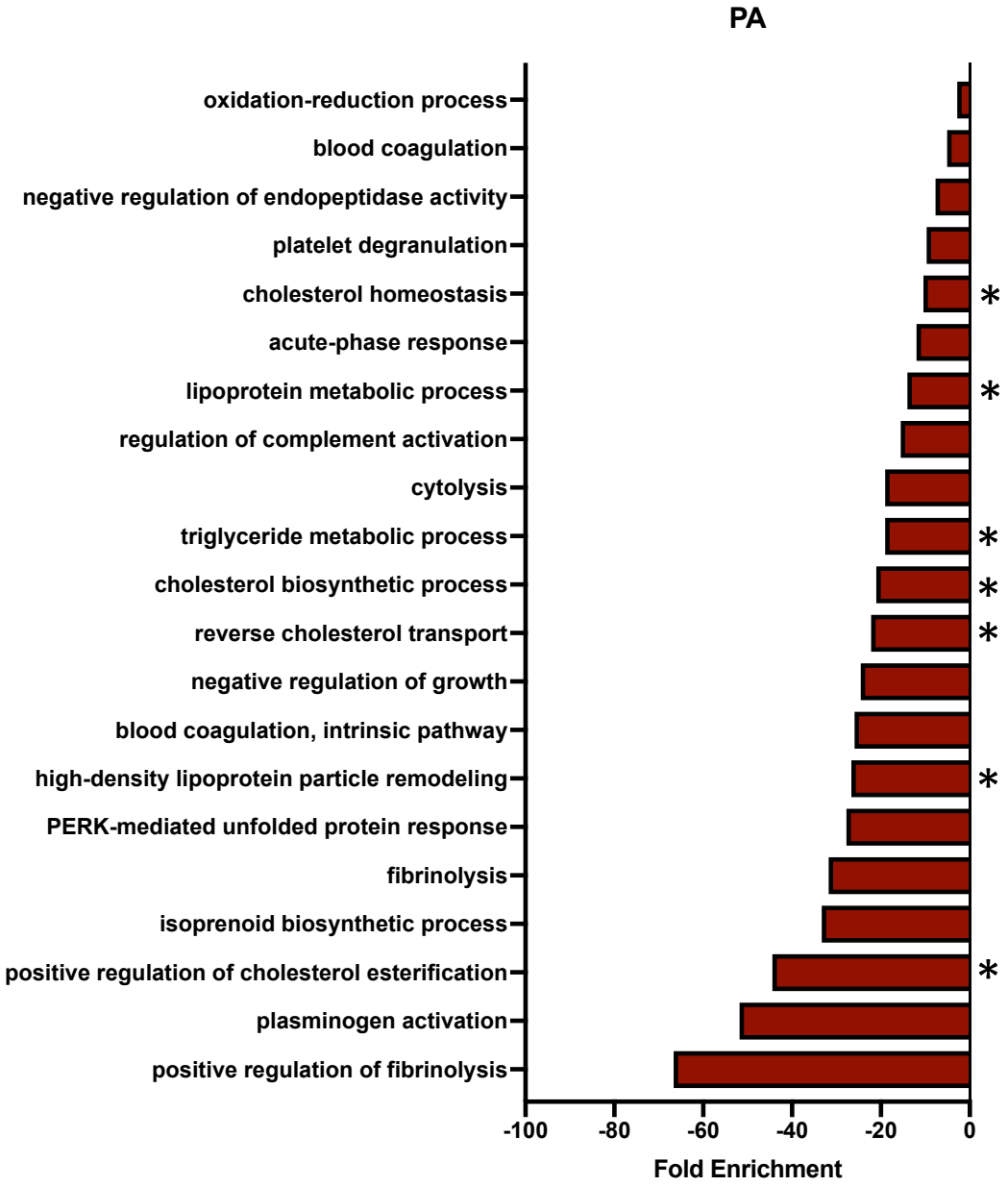

Figure 6.3 Gene ontology enrichment analysis for PNPLA3^{KO} and PNPLA3^{UC} cells treated with palmitic acid.

Gene ontology enrichment analysis comparing PNPLA3^{UC} and PNPLA3^{KO} cells treated with palmitic acid. Statistically significantly upregulated pathways are shown in green and downregulated pathways are shown in red. Metabolic processes are marked with a star. These results indicate that PNPLA3^{KO} cells downregulate several metabolic pathways compared to PNPLA3^{UC} cells regardless of treatment type.

The results of the gene ontology analysis can be found in Figures 6.1-6.3 where upregulated pathways are shown in green and downregulated pathways are shown in red.

We found that, regardless of treatment type, the vast majority of differentially enriched pathways were downregulated in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. The small number of upregulated pathways related to cell proliferation and migration, but the enrichment of these pathways was relatively small. In general, the downregulated pathways involved metabolism, innate immunity, inflammation, and cell-stress responses. The affected metabolic pathways generally related to the homeostasis of triglycerides, cholesterol, and lipoproteins though pathways involved in the metabolism of other lipid species such as retinoids and fatty acids were also represented. This general downregulation of lipid metabolism is in accordance with our previous results which show that PNPLA3-edited cells favour triglyceride formation over upregulation of metabolic processes in response to lipid stressors.

Consistent with the reduced cytotoxicity of palmitic acid in PNPLA3^{KO} cells, we found that cellular pathways responsible for responding to cell stress were downregulated in palmitic acid treated PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. Specifically, the PERK-mediated unfolded protein response pathway was downregulated in these cells. This data further supports our hypothesis that PNPLA3-edited cells escape palmitic acid-induced lipotoxicity by downregulating lipid metabolic pathways in favour of triglyceride formation. Though consistent with our previous experiments, these data remain difficult to reconcile with the worsening disease phenotype seen in carriers of the PNPLA3 I148M variant.

In addition, several pathways involved in innate immunity and inflammation were downregulated in the PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. This result was surprising given that the I148M variant is associated with worsening disease and high necro-inflammatory scores. However, the innate immune response system that was most profoundly affected in the PNPLA3-edited cells was the complement system. The complement system is an innate immune process that plays a significant role in defending the host from pathogens. However, recent evidence suggests that it also plays a significant role in metabolic homeostasis [269]. The complement factor C3 plays a significant role in regulating hepatic steatosis. Mice deficient in C3 have marked hepatic steatosis due to increased triglyceride accumulation and reduced fatty acid oxidation [270]. Therefore,

the reduced expression of genes relating to the complement system, including C3, in PNPLA3^{KO} cells could be contributing to the observed phenotype of increased lipid accumulation and decreased lipid metabolism in these cells.

However, progression from NAFLD to NASH has been linked to increased hepatic deposition of activated complement factors C3 and C9 [271]. Therefore, the reduction in expression of genes involved in the complement system as well as other inflammatory and cell stress pathways remains antithetical to the role of the I148M variant in worsening disease. There are several explanations for this discrepancy. It is possible that our *in vitro* system does not allow enough time for an inflammatory response to be mounted in response to metabolic dysregulation. NAFLD is a disease that requires decades to develop and progress from simple steatosis through end-stage liver disease. Thus, the full spectrum of the disease is unlikely to be captured in our system after just 24 hours to one week of lipid treatment. It remains possible that longer treatment durations with lipids or prolonged metabolic dysregulation could lead to the upregulation of pathways involved in the inflammatory response. Alternately, these data could indicate that the inflammatory stimuli for disease progression in patients with the I148M variant may originate from cells other than hepatocytes. NAFLD is a multicellular disease that requires complex intercellular signalling between all hepatic cell types. Given that HSCs have been implicated in the inflammatory response in NAFLD and the I148M variant exacerbates the inflammatory phenotype of these cells, it is possible that the I148M variant exerts its inflammatory phenotype through HSCs rather than hepatocytes [113, 166]. Additionally, it remains possible that hepatocytes carrying the risk allele may secrete factors that propagate an immune response in Kupffer cells or other liver resident immune cells.

Given that the majority of differentially enriched pathways between PNPLA3^{KO} and PNPLA3^{UC} cells were downregulated, we wanted to understand the nature of this downregulation. The gene ontology enrichment analysis only examines relative gene expression between PNPLA3^{KO} and PNPLA3^{UC} cells. Based upon this analysis, it is difficult to understand whether PNPLA3^{KO} cells truly downregulate these pathways or if they fail to properly upregulate these pathways in response to lipid supplementation. In

order to assess this, we performed principle component analysis comparing the three treatments, control, oleic acid, and palmitic acid, within each genotype (Figure 6.4).

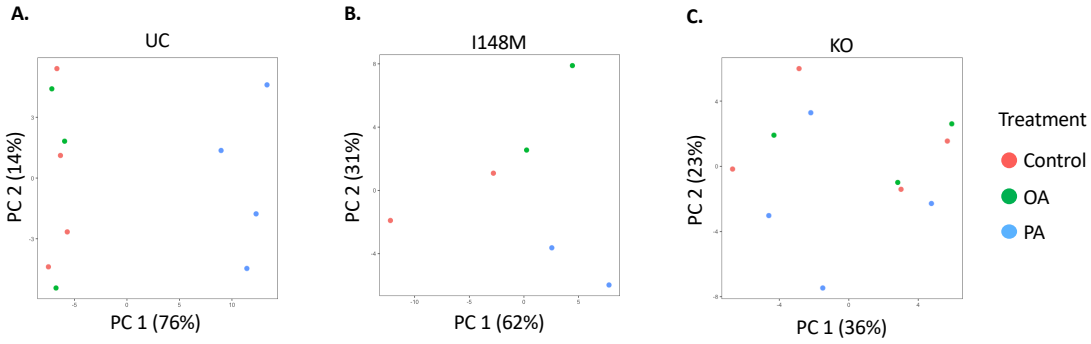

Figure 6.4 PCA plot of treatments by genotype.

PCA plot comparing transcriptomic differences between the three treatments within **A.** PNPLA3^{UC} cells, **B.** PNPLA3^{I148M} cells, and **C.** PNPLA3^{KO} cells (PNPLA3^{UC}: n = 2 clones and 2 independent experiments; PNPLA3^{I148M}: n = 1 clone and 2 independent experiments; PNPLA3^{KO}: n = 2 clones and 2 independent experiments). In PNPLA3^{UC} cells, the control and oleic acid treated groups cluster together while the palmitic acid treated group clusters far apart. However, treatment type does not seem to profoundly affect the transcriptomic profile of PNPLA3^{I148M} or PNPLA3^{KO} cells which indicates that these cells may not be activating the proper metabolic pathways when presented with a lipid stressor.

We found that the PNPLA3^{UC} cells demonstrated significant transcriptomic differences between treatments. The PNPLA3^{UC} cells treated with control and oleic acid medium clustered together while the palmitic acid treated cells clustered separately. The variability between palmitic acid treated cells and the other treatments accounted for 76% of the variability in this analysis. This observation is supported by the number of differentially expressed genes between the treatment groups. In PNPLA3^{UC} cells treated with oleic acid, there were only 31 differentially expressed genes compared to control treated cells. However, when PNPLA3^{UC} cells were treated with palmitic acid, there were 1,826 differentially expressed genes. These profound transcriptomic differences are in accordance with the stark lipotoxic phenotype observed in palmitic acid treated cells of this genotype. Alternatively, the transcriptomic profiles of PNPLA3^{I148M} and PNPLA3^{KO} cells failed to separate based upon treatment alone. In both treatments, there were very few differentially expressed genes in PNPLA3^{I148M} (OA: 40; PA: 93) and PNPLA3^{KO} (OA:27; PA: 75) cells compared to control treated cells in each genotype. These data indicate that PNPLA3-edited cells fail to upregulate lipid metabolic processes when

presented with an influx of fatty acids. As suspected the PNPLA3^{I148M} and PNPLA3^{KO} cells fail to differentiate between MUFAs and SFAs and relegate them both to the same metabolic fate: esterification into triglycerides. In lieu of the appropriate metabolic response to each class of fatty acid, PNPLA3 edited cells divert the exogenous fatty acids into triglyceride storage which results in increased steatosis and reduced SFA-induced lipotoxicity.

There is significant overlap between lipid metabolic processes, especially those for cholesterol transport/metabolism, and drug metabolism. Given the lower expression of genes involved in lipid metabolism in the PNPLA3^{KO} cell compared to PNPLA3^{UC} cells, we wanted to examine if *PNPLA3* genotype has an effect on drug metabolism as well. We hypothesized that PNPLA3-edited cells may downregulate their drug metabolism concomitantly with lipid metabolism. Since downregulation of DMEs has been linked to worsening disease phenotype in NAFLD patients, we believe that this could represent the link between the I148M variant and worsening disease phenotypes. Tables 6.1 through 6.3 show that PNPLA3^{KO} cells treated with control medium had lower expression of genes involved in all four phases of drug metabolism compared to PNPLA3^{UC} cells treated with the same medium.

Enzyme	Type	Phase	LFC	FDR
ADH1A	Alcohol Dehydrogenase	Phase 1	-6.2256	4.92E-11
ADH1B	Alcohol Dehydrogenase	Phase 1	-5.3291409	1.19E-05
ADH5	Alcohol Dehydrogenase	Phase 1	-0.283764	0.03026105
ADH6	Alcohol Dehydrogenase	Phase 1	-4.0456059	6.30E-13
ALDH1A1	Aldehyde Dehydrogenase	Phase 1	-0.680466	1.38E-06
ALDH1A3	Aldehyde Dehydrogenase	Phase 1	1.10719819	5.02E-09
ALDH1L2	Aldehyde Dehydrogenase	Phase 1	-1.8362438	0.04998308
ALDH4A1	Aldehyde Dehydrogenase	Phase 1	-3.6080842	1.60E-09
ALDH5A1	Aldehyde Dehydrogenase	Phase 1	-0.7758045	0.00010471
ALDH6A1	Aldehyde Dehydrogenase	Phase 1	-0.7463205	0.00066543
ALDH7A1	Aldehyde Dehydrogenase	Phase 1	-0.7553237	2.03E-06
ALDH8A1	Aldehyde Dehydrogenase	Phase 1	-1.8004226	1.11E-10
ALDH9A1	Aldehyde Dehydrogenase	Phase 1	-0.3717261	0.00319375
CYP19A1	Cytochrome P450	Phase 1	-3.1671584	2.73E-29

CYP1A1	Cytochrome P450	Phase 1	-0.806655	0.09241302
CYP1B1	Cytochrome P450	Phase 1	-0.5165573	0.00252969
CYP2B7P	Cytochrome P450	Phase 1	0.87391532	0.00574331
CYP2C8	Cytochrome P450	Phase 1	-3.7677259	1.04E-16
CYP2C9	Cytochrome P450	Phase 1	-1.3121207	0.05168297
CYP2J2	Cytochrome P450	Phase 1	-1.3578995	0.0001618
CYP39A1	Cytochrome P450	Phase 1	-1.9488667	5.65E-09
CYP3A7	Cytochrome P450	Phase 1	-6.7157076	1.61E-19
CYP4A11	Cytochrome P450	Phase 1	-3.717564	1.37E-06
CYP4V2	Cytochrome P450	Phase 1	-1.0090765	2.10E-07
CYP7A1	Cytochrome P450	Phase 1	-1.9578987	0.00156614
CYP8B1	Cytochrome P450	Phase 1	-1.0605197	5.93E-07

Table 6.1 List of differentially expressed phase one DMEs.

Phase one DMEs differentially expressed between PNPLA3^{UC} and PNPLA3^{KO} cells. The enzymes are divided by the enzyme family they belong to and the log fold change (LFC) and false discovery rate (FDR) are listed for each differentially expressed enzyme.

Table 6.1 shows the significantly differentially expressed phase one DME genes between PNPLA3^{KO} and PNPLA3^{UC} genes. Expression of *ADH1*, the gene responsible for oxidizing ethanol into acetaldehyde, had a more than five-fold lower expression in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. Since the I148M variant is implicated in more severe ALD phenotypes, this finding is extremely interesting. Additionally, we saw a stark downregulation of nearly all of the *CYP* family genes that were differentially expressed in our system. Of note, the *CYP3A7* gene showed a greater than six-fold decrease in expression in PNPLA3^{KO} cells. CYP3A7 is the foetal isoform of CYP3A4 which is the most prolific phase one DME responsible for nearly 50% of all drug metabolism in hepatocytes [259]. Given that the HLCs have a phenotype more closely resembling foetal cells than adult cells, CYP3A7 likely assumes the role of CYP3A4 in our system. The profound downregulation of this gene in PNPLA3^{KO} cells indicates that these cells have severely impaired drug metabolism. This data is also consistent with the data from Chapter 3 showing PNPLA3^{KO} cells in the A1ATDR/R background have lower basal CYP3A4 activity, though this difference did not reach statistical significance. Based

upon these data, it appears that PNPLA3-edited cells have impaired drug metabolism, and this is a direct result of their genotype rather than altered differentiation capacity.

We next examined the expression of phase two DMEs in Table 6.2. We examined differentially expressed genes belonging to three classes of phase two DMEs: *UGTs, SULTs*, and *GSTs*. We found that, in general, *UGTs* and *SULTs* were downregulated in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. However, the relative expression difference in these enzymes was much smaller than seen in the phase one enzymes. Interestingly, of the four *GST* enzymes that were differentially expressed, three were upregulated in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. This would indicate that PNPLA3^{KO} cell may be more resistant to oxidative stress due to their high capacity to conjugate oxidized molecules to the antioxidant glutathione. Despite this anomaly, the general downregulation of key phase two DMEs supports the hypothesis that loss of PNPLA3 function leads to a reduced functionality of drug metabolism in hepatocytes.

Enzyme	Type	Phase	LFC	FDR
GSTA1	Glutathione S-Transferase	Phase 2	1.22859997	1.27E-07
GSTA4	Glutathione S-Transferase	Phase 2	-0.5785737	0.00102473
GSTM4	Glutathione S-Transferase	Phase 2	0.48543491	0.03224733
GSTP1	Glutathione S-Transferase	Phase 2	0.26516017	0.09518873
SULT1A1	Sulfotransferase	Phase 2	0.64732791	0.00050337
SULT1C2	Sulfotransferase	Phase 2	-0.7774563	0.00808405
SULT1E1	Sulfotransferase	Phase 2	-1.5830545	0.00160952
UGT2A3	Uridine Diphospho Glucuronosyltransferase	Phase 2	0.61115064	0.06711183
UGT2B10	Uridine Diphospho Glucuronosyltransferase	Phase 2	-2.0847303	7.98E-06
UGT2B17	Uridine Diphospho Glucuronosyltransferase	Phase 2	1.14170542	0.03227578
UGT2B28	Uridine Diphospho Glucuronosyltransferase	Phase 2	-1.0059826	0.05619861
UGT2B4	Uridine Diphospho Glucuronosyltransferase	Phase 2	-2.0253223	2.00E-15
UGT2B7	Uridine Diphospho	Phase 2	0.57236177	0.00148831

	Glucuronosyltransferase			
UGT3A1	Uridine Diphospho Glucuronosyltransferase	Phase 2	-0.7236335	0.08331915
UGT8	Uridine Diphospho Glucuronosyltransferase	Phase 2	0.68452334	0.06832374

Table 6.2 List of differentially expressed phase two DMEs.

Phase two DMEs differentially expressed between PNPLA3^{UC} and PNPLA3^{KO} cells. The enzymes are divided by the enzyme family they belong to and the log fold change (LFC) and false discovery rate (FDR) are listed for each differentially expressed enzyme.

Finally, we examine the expression of transporters responsible for the influx (phase zero DMEs) of drugs and the efflux (phase three DMEs) of drug metabolites in Table 6.3. Once again there was a general down regulation of both phase zero and phase three DMEs in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. While decreasing the expression of drug importers is a cytoprotective process to prevent the import of unmetabolizable toxins into hepatocytes, the downregulation of exporters could increase hepatocellular damage by increasing the exposure time of hepatocytes to toxic substances.

Enzyme	Type	Phase	LFC	FDR
SLC10A1	Solute Carrier Transporter Family	Phase 0	-0.8274799	0.01063994
SLC22A1	Solute Carrier Transporter Family	Phase 0	-2.5726521	1.44E-05
SLC22A7	Solute Carrier Transporter Family	Phase 0	-1.6193629	7.40E-18
SLCO1B1	Solute Carrier Organic Anion Transporter Family	Phase 0	-5.4283323	5.78E-08
ABCB1	ATP Binding Cassette Transporter	Phase 3	-1.4563573	0.01747606
ABCG2	ATP Binding Cassette Transporter	Phase 3	0.62407264	0.03966208
ABCG8	ATP Binding Cassette Transporter	Phase 3	-0.4650829	0.05316166

Table 6.3 List of differentially expressed drug transporters (phase zero/phase three DMEs).

Phase zero and phase three DMEs differentially expressed between PNPLA3^{UC} and PNPLA3^{KO} cells. The enzymes are divided by the enzyme family they belong to and the log fold change (LFC) and false discovery rate (FDR) are listed for each differentially expressed enzyme.

In all, the RNA sequencing analysis indicates that PNPLA3-edited cells globally downregulate both lipid and drug metabolic pathways. Gene ontology enrichment analyses indicated that PNPLA3^{KO} cells downregulate metabolic pathways involved in

triglyceride, fatty acid, cholesterol, and lipoprotein homeostasis compared to PNPLA3^{UC} cells. This lower expression of genes involved in metabolic processes is maintained even after treatment with lipid stimuli such as oleic acid or palmitic acid. The PNPLA3^{I148M} and PNPLA3^{KO} cells did not exhibit differential transcriptomic responses to oleic acid and palmitic acid unlike the PNPLA3^{UC} cells. Therefore, these cells have incompetent metabolic responses regardless of external stimuli supporting the evidence from previous chapters that PNPLA3-edited cells accumulate more lipid droplets and fail to properly respond to lipid-induced stress. This metabolic dysregulation has the potential to affect the long-term health of these hepatocytes; however, given the short duration of treatment in our system, we failed to observe any maladaptive effects on our cells.

PNPLA3^{KO} cells also have lower expression of genes involved in innate immunity, inflammation, and cell stress. This downregulation is difficult to reconcile with the worsening disease phenotype and higher necro-inflammatory scores of patients with the I148M variant. The innate immunity pathway that is most closely linked to alterations in *PNPLA3* genotype is the complement pathway which has been implicated in triglyceride homeostasis as well. Therefore, in our cells, perturbations in the complement system are likely linked to its role in metabolism rather than the innate immune response.

Given the global downregulation of metabolic pathways, we hypothesized that drug metabolism may also be negatively affected in PNPLA3-edited cells which offers an explanation for the higher disease burden in carriers of the risk allele. We found that several DMEs from all phases of drug metabolism were differentially expressed in our system. In accordance with the trends seen in lipid metabolism, all phases of drug metabolism were downregulated in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. This trend was especially pronounced in the phase one DMEs that are the first line of defence against toxic drugs and lipid species. Given these findings, we believe that the I148M variant, at least in part, exerts its deleterious effects on NAFLD progression by interfering with the detoxification of exogenous xenobiotics as well as endogenous lipid species.

6.3 PNPLA3 edited cells may be more susceptible to other forms of hepatotoxicity

In addition to its role in metabolic diseases such as NAFLD, the I148M variant in PNPLA3 has also been linked to diseases of hepatic detoxification such as alcoholic liver disease, hemochromatosis, and chemotherapy-induced liver injury [139, 143, 224, 225]. Given this clinical data and the global downregulation of DMEs in PNPLA3^{KO} cells, we hypothesized that this incompetent drug metabolism makes PNPLA3-edited cells more susceptible to hepatotoxic insults. Analogously, it can be hypothesized that carriers of the I148M variant have more liver damage than non-carriers due to their inability to detoxify common hepatotoxic metabolites such as ethanol. This hypothesis presents two distinct pathways for increased steatosis and hepatic injury contributing to NASH progression in these patients. This notion is supported by data from our system that increased steatosis in PNPLA3-edited cells does not contribute to lipotoxic cell injury or disease progression. In order to test this hypothesis, we subjected the cells in our system to three common hepatotoxic insults and assessed the differential response of the *PNPLA3* genotypes to these insults. We differentiated PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells from the FSPS13B background into HLCs and placed them into 3D culture. In order to assess the effect of steatosis on toxicity, we then treated the HLCs with either control medium or medium supplemented with oleic acid for 48 hours. Following fatty acid treatment, the HLCs were then subjected to treatment with ethanol, iron, or acetaminophen (paracetamol) to induce toxicity. The extent of toxicity was then measured using viability analyses. We chose these three toxic compounds because they have been previously verified in *in vitro* culture systems in our lab. They are toxins that humans encounter in sublethal doses on a regular basis, and PNPLA3 has been connected to diseases caused by failure to detoxify both ethanol and iron, namely ALD and hemochromatosis. We hypothesized that PNPLA3^{I148M} and PNPLA3^{KO} cells treated with the three toxic insults would have lower viability than PNPLA3^{UC} cells. Since NAFLD has been proven to increase the risk of hepatotoxicity and drug induced liver injury, we also hypothesized that higher steatosis in oleic acid treated cells would increase the toxicity of these insults in all three genotypes.

Figure 6.5a shows a schematic of the ethanol toxicity treatment. Differentiated HLCs of all three genotypes were first treated for 48 hours with either control medium or oleic acid medium to induce steatosis. These lipid-loaded cells were then treated for 48 hours with 100 mM ethanol. Cells grown *in vitro* are less susceptible to ethanol toxicity, so the ethanol treatment was followed by treatment with 20 mM TNFα for 24 hours to exacerbate toxicity [272]. Figure 6.5b shows the effect of this treatment on the viability of HLCs from the three genotypes. This dose of ethanol was relatively non-toxic to PNPLA3^{UC} cells and had very little effect on the viability of control or oleic acid treated cells of this genotype. However, the ethanol and TNFα treatment resulted in significantly lower viability of PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. The PNPLA3^{I148M} cells had lower viability than their PNPLA3^{UC} counterparts but this difference did not reach statistical significance. This result verifies the RNA sequencing data shown in Table 6.1 which shows that the major enzymes involved in ethanol metabolism, *ADH1A, ADH1B*, and *ALDH1A1*, are all significantly downregulated in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells. Due to the lower expression of these detoxifying enzymes, even ethanol concentrations that were relatively nontoxic to PNPLA3^{UC} cells were quite harmful to the PNPLA3^{KO} cells. Given these data, NALFD patients with the I148M variant who consume even very small amounts of alcohol may be at risk of significant liver damage which contributes to the progression of the disease toward NASH. Interestingly, we found that induction of steatosis using oleic acid did not exacerbate the ethanol toxicity in any of the genotypes. These data support the hypothesis that steatosis and the progressive liver damage seen in NASH are functionally separate processes in carriers of the I148M variant.

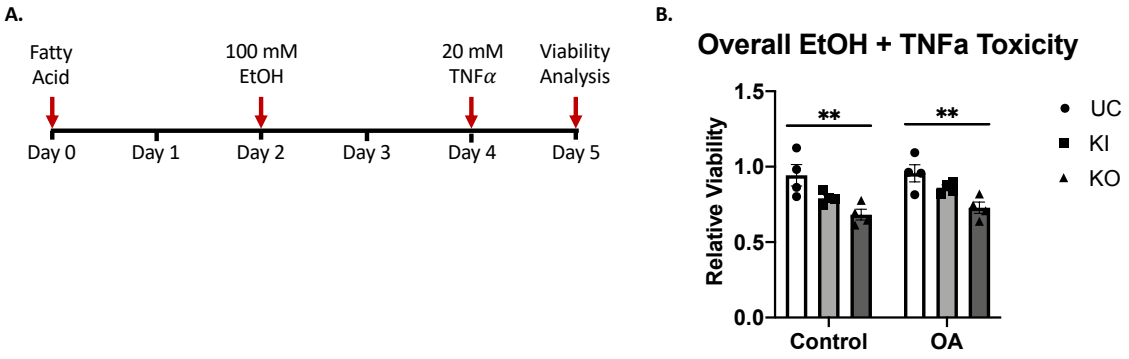

Figure 6.5 Ethanol Toxicity.

A. Timeline of ethanol and TNFα treatment to induce toxicity. **B.** Relative viability of ethanol + TNFα treated cells to untreated cells within each genotype ($PNPLA3^{UC}$: n = 2 clones and 2 independent experiments; $PNPLA3^{I148M}$: n = 2 clones and 2 independent experiments; $PNPLA3^{KO}$: n = 2 clones and 2 independent experiments)
. Given the sublethal dose of ethanol, $PNPLA3^{UC}$ cell maintained high viability following treatment. Conversely, $PNPLA3^{KO}$ cells showed an acute toxicity when treated with even a sublethal dose of ethanol. $PNPLA3^{I148M}$ cells were intermediate between $PNPLA3^{UC}$ and $PNPLA3^{KO}$ cells. This trend was consistent in both control and oleic acid treated cells indicating that this effect may be independent of steatosis. Ordinary one-way ANOVAs with Dunnett's multiple comparisons tests were performed to test statistical significance between means. Error bars represent SEM.

In addition to NAFLD and ALD, the I148M variant in PNPLA3 has been linked to worsening disease phenotypes in patients with hereditary hemochromatosis. Therefore, we hypothesized that PNPLA3-edited cells would be more susceptible to iron-induced toxicity. Figure 6.6a shows the timeline of iron treatment. As before, HLCs of each genotype were treated for 48 hours with either control or oleic acid medium followed by a 24-hour treatment with 200 μM ferric ammonium citrate (FAC). The viability analysis in Figure 6.6b indicates that this iron treatment was non-toxic in all three genotypes. This lack of toxicity is likely due to impaired iron uptake in our system. The 3D culture medium used in these treatments is chemically defined and does not contain foetal bovine serum; additionally, the concentration of transferrin is quite low. Thus, there was likely very little import of the FAC into the HLCs. However, there was a slight decrease in viability in all three genotypes pre-treated with oleic acid which indicates that at least small concentrations of FAC were entering the cells and that steatosis sensitizes these cells to ferroptosis. Unlike the ethanol treatment, no inter-genotypic differences were observed in the viability of iron treated cells. These data indicate that iron alone may not be specifically toxic to carriers of the I148M variant. The role of the I148M variant in inherited hemochromatosis may rely on the interaction between the I148M variant in PNPLA3 and the C282Y mutation in the HFE gene that causes hereditary hemochromatosis [143]. Thus, our system may not be the ideal platform to examine the role of the PNPLA3 I148M variant in iron toxicity.

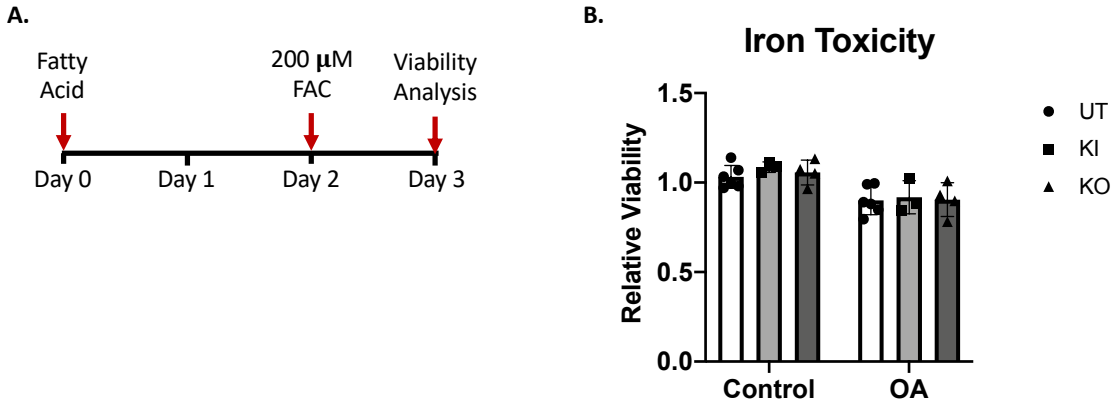

Figure 6.6 Iron Toxicity.

A. Timeline of ferric ammonium citrate (iron) treatment. **B.** Relative viability of FAC treated cells to untreated cells within each genotype ($PNPLA3^{UC}$: n = 2 clones and 3 independent experiments; $PNPLA3^{I148M}$: n = 2 clones and 3 independent experiments; $PNPLA3^{KO}$: n = 2 clones and 3 independent experiments). FAC induced very little toxicity and there was no notable difference in viability between PNPLA3 genotypes. Ordinary one-way ANOVAs with Dunnett's multiple comparisons tests were performed to test statistical significance between means. Error bars represent SEM.

We next examined the effect of PNPLA3 genotype on the toxicity of the common over-the-counter drug acetaminophen, also known as paracetamol. The I148M variant has not been associated with increased acetaminophen toxicity clinically, but we chose to use this drug as a surrogate for drug toxicity in our system. Figure 6.7a shows the treatment schematic for the acetaminophen toxicity experiment. We treated the HLCs of each genotype with either control or oleic acid medium for 48 hours followed by treatment with 25 mM acetaminophen for 48 hours. Figure 6.7b shows that acetaminophen treatment induced strong toxicity in all three genotypes. There was no significant difference in viability between the three genotypes or between control or oleic acid treated cells of the same genotype. To ensure that the high toxicity of this dose was not obscuring intergenotypic differences, we tested a sublethal dose (12.5 mM acetaminophen) and saw a similar trend (data not shown). These results, while initially contradictory to the ethanol toxicity results, are supported by the RNA sequencing data. The majority of acetaminophen is metabolized through glucuronidation and sulfation; however, at high concentrations, these detoxification pathways are overwhelmed, and acetaminophen is instead metabolized through a combination of oxidation by CYP2E1 and glutathione

conjugation by GST enzymes [273]. The RNA sequencing data indicates that while the PNPLA3^{KO} cells have lower expression of both *UGT* and *SULT* enzymes, these cells express higher levels of GST enzymes. There was no significant difference in *CYP2E1* expression between PNPLA3^{KO} and PNPLA3^{UC} cells. Based upon these data, it is conceivable that PNPLA3^{KO} cells may have a lower capacity for detoxification of acetaminophen through glucurondation and sulfation but their increased glutathione transferase capabilities compensate for these deficiencies. Thus, the overall viability of the cells remained unchanged, but the process of detoxification may differ between PNPLA3-edited cells and their untargeted control counterparts. Additional experiments are needed to examine the veracity of this hypothesis.

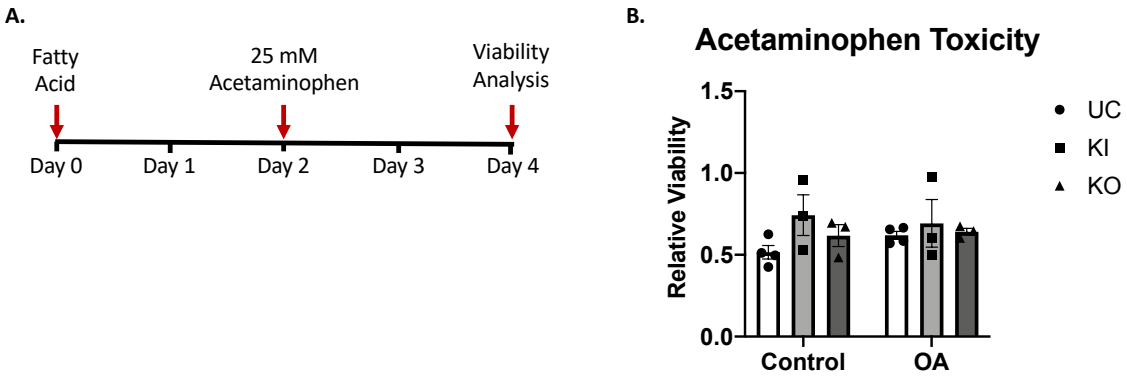

Figure 6.7 Acetaminophen toxicity.

A. Timeline of acetaminophen treatment to induce toxicity. **B.** Relative viability of acetaminophen treated cells to untreated cells within each genotype (PNPLA3^{UC}: n = 2 clones and 2 independent experiments; PNPLA3^{I148M}: n = 2 clones and 2 independent experiments; PNPLA3^{KO}: n = 2 clones and 2 independent experiments). Acetaminophen caused acute toxicity in all cells. There were no significant differences in viability between PNPLA3 genotypes or treatment groups. Ordinary one-way ANOVAs with Dunnett's multiple comparisons tests were performed to test statistical significance between means. Error bars represent SEM.

We used three *in vitro* toxicity assays to examine the hypothesis that downregulation of DMEs in PNPLA3-edited cells leads to increased susceptibility to hepatotoxicity in these cells. Additionally, we sought to determine if steatosis was a contributing factor to this hepatotoxicity by pre-treating the cells subjected to toxic insult with oleic acid to induce lipid accumulation in these cells. Induction of toxicity with ethanol and TNFα, FAC, and acetaminophen had differential results in our system. Treatment with ethanol and TNFα

induced a significantly higher toxicity in PNPLA3^{KO} cells than PNPLA3^{UC} cells while PNPLA3^{I148M} cells demonstrated an intermediate phenotype. This effect was seen at ethanol concentrations that were sublethal to PNPLA3^{UC} cells. These results are consistent with a role for the PNPLA3 I148M variant in exacerbating ALD. However, treatment with iron and acetaminophen failed to produce inter-genotypic differences in toxicity. There was no difference between control and oleic acid treated cells in any of the toxicity challenges indicating that susceptibility to hepatotoxicity is dissociated from the presence of steatosis. The data from these three toxicity treatments are inconsistent making it difficult to conclude if loss of PNPLA3 activity contributes to hepatocellular damage by increasing the susceptibility of these cells to common hepatotoxins. Additional studies will be needed to fully understand the role that PNPLA3 plays in drug metabolism. That being said, the results from the RNA sequencing as well as the preliminary results from the ethanol toxicity experiment still offer validity to this hypothesis.

6.4 Conclusions

In this chapter, we sought to explore the mechanisms by which changes to *PNPLA3* genotype affect susceptibility to hepatic injury. We examined the transcriptomic differences between PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells using principle component analyses, differential gene expression analyses, and gene ontology enrichment analyses. We found that PNPLA3^{KO} cells had a general reduction in expression of metabolic genes compared to PNPLA3^{UC} cells, especially those involved in the homeostasis of triglycerides, fatty acids, cholesterol, and lipoproteins. This trend was independent of fatty acid treatment and offers insight into how PNPLA3-edited cells accumulate more lipid droplets than their PNPLA3^{UC} counterparts. Principle component analyses indicated that, unlike PNPLA3^{UC} cells, PNPLA3-edited cells failed to differentiate between control, oleic acid, and palmitic acid treatment in their transcriptional profile. This data offers further credence to the hypothesis that PNPLA3-edited cells fail to metabolically differentiate between SFAs and MUFAs. The global downregulation of metabolism in PNPLA3^{KO} cells compared to PNPLA3^{UC} cells extended beyond lipid metabolism to drug metabolism as well. DMEs from every phase of the detoxification cascade were downregulated in PNPLA3^{KO} cells compared to

PNPLA3^{UC} cells. These data led us to hypothesize that alterations to PNPLA3 may sensitize HLCs to other forms of hepatotoxicity beyond lipotoxicity. We subjected PNPLA3^{UC}, PNPLA3^{I148M}, and PNPLA3^{KO} cells to treatment with ethanol and TNFα, iron, and acetaminophen to induce cytotoxicity. We found that PNPLA3^{KO} cells were more susceptible to ethanol-induced toxicity compared to PNPLA3^{UC} cells, even at low concentrations that were sublethal to the PNPLA3^{UC} cells. However, we failed to replicate these findings in the iron and acetaminophen toxicity assays. Additional studies are needed to determine if carriers of the I148M variant are more susceptible to other forms of hepatotoxicity beyond ethanol. In all, we found that loss of PNPLA3 expression leads to a global downregulation in metabolism. This metabolic dysregulation could have adverse consequences on the long-term health of the hepatocyte either by inducing toxicity or inflammation. Additional toxicity studies as well as long-term culture studies will be needed to fully elucidate the consequences of reduced PNPLA3 expression and metabolic activity on these cells.