

*INDUCED PLURIPOTENT STEM CELL
DERIVED LIVER MODEL FOR THE STUDY
OF PNPLA3-ASSOCIATED NON-
ALCOHOLIC FATTY LIVER DISEASE*



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DECLARATION

This thesis is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text. It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my thesis has already been submitted, or, is being concurrently submitted for any such degree, diploma, or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text.

In accordance with the Degree Committee for the Faculty of Biology guidelines, this thesis is does not exceed 60,000 words in length (excluding figures, photographs, tables, appendices, and bibliography).

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SUMMARY

Non-alcoholic fatty liver disease (NAFLD) is now the leading cause of chronic liver disease in the developed world, afflicting approximately one in four adults globally. NAFLD is defined by the accumulation of fat within the liver which ranges in severity from simple steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis, and hepatocellular carcinoma. Until recently, NAFLD has been considered to be a consequence of metabolic syndrome; however, recent studies suggest that genetic factors may also influence disease onset and progression. Accordingly, genome wide association studies have linked the I148M variant in the gene coding for Patatin-like phospholipase domain-containing protein 3 (PNPLA3) with NAFLD aggravation without underlying metabolic disease. However, despite over a decade of research, the function of PNPLA3 and its role in the pathogenesis of NAFLD remains largely obscure. The lack of clarity regarding the function and disease associations of PNPLA3 is due in large part to the lack of a comprehensive human model of PNPLA3-associated NAFLD. In order to address this need, we have developed an *in vitro* model that takes advantage of the unique properties of human induced pluripotent stem cells (hiPSC) and the CRISPR/CAS9 gene editing technology.

We first used CRISPR/CAS9 to generate hiPSC lines with either a knock-out (PNPLA3^{KO}) of the *PNPLA3* gene or with the I148M variant knocked in (PNPLA3^{I148M}). The resulting cells were then differentiated into hepatocytes and grown in 3D culture conditions to improve their maturity and functionality. The differentiated cells were treated with either monounsaturated or saturated free fatty acids to induce NAFLD-like phenotypes (lipid accumulation and lipid toxicity, respectively) and characterized by various functional, genomic, and lipidomic assays. The genetically edited sublines showed similar differentiation efficiency toward hepatocytes as untargeted cells indicating that changes in PNPLA3 activity does not affect hepatic development. Following treatment with free fatty acids, PNPLA3^{KO} cells showed higher lipid accumulation than untargeted cells as well as an altered pattern of response to lipid-induced stress. Indeed, PNPLA3^{KO} cells were resistant to saturated fatty acid-induced lipotoxicity. Furthermore, lipidomic analyses suggest that the PNPLA3 edited cells may be avoiding cell death by shuttling the saturated fatty acids into triglycerides rather than other metabolic pathways. These findings were initially incongruent with the human

disease; however, despite their resistance to lipid induced stress, the PNPLA3^{KO} cells downregulated all phases of drug metabolizing enzymes which made them more susceptible to other forms of hepatotoxicity such as ethanol. The PNPLA3^{I148M} cells exhibited an intermediate phenotype between untargeted and PNPLA3^{KO} cells.

These results demonstrate for the first time in a fully human model that the I148M variant in PNPLA3 is a loss of function variant. This loss of PNPLA3 activity leads to the global downregulation of metabolic pathways likely due to the sequestration of fatty acids in triglycerides caused by reduced lipid droplet remodelling capacity in these hepatocytes. Our results indicate that patients carrying the I148M variant have lower hepatic metabolic activity which causes steatosis, reduced susceptibility to lipotoxicity, and increased susceptibility to other forms of hepatotoxicity which may contribute to NAFLD progression. This novel system provides the first opportunity to study the role of PNPLA3 in the development and progression of human NAFLD *in vitro*.

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Winston Churchill once said, “Success is stumbling from failure to failure with no loss of enthusiasm.” I feel this quote perfectly summarizes the experience of obtaining a PhD. Most days, science is choosing to move forward in the face of immanent failure. I don’t think that I can claim that I never lost any enthusiasm throughout this process; however, this thesis is a testament that even though my PhD journey was, by no means, an unadulterated success, I did manage to fail in the right direction. There are so many people who have contributed to my journey in large and small ways. This would never have been possible without each and every one of you.

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LIST OF ABBREVIATIONS AND ACRONYMS

- A1AT: Alpha-1-anti-trypsin
ABC: ATP-binding cassette
ACC: Acetyl-CoA carboxylase
ACLY: ATP citrate lyase
ADH: Alcohol dehydrogenase
AGPAT: Acyl-sn-glycerol-3-phosphate acyltransferase
AKT: RAC-alpha serine/threonine protein kinase
ALB: Albumin
ALD: Alcoholic liver disease
ALDH: Aldehyde dehydrogenase
AP-1: Activator protein 1
APOB-100: Apolipoprotein B-100
Asp: Aspartic acid
ATF6: Activating transcription factor 6
ATP: Adenosine triphosphate
BAX: Bcl-2-associated X protein
BIP: Binding immunoglobulin protein
BMP4: Bone morphogenetic protein 4
BODIPY: Boron-dipyrromethene
c-MYC: MYC proto-oncogene
C3: Complement component 3
C9: Complement component 9
CAR: Constitutive androstane receptor
CAS9: CRISPR-associated protein 9
CAT: carnitine-acylcarnitine translocase
CCL2: Chemokine ligand 2
CCL5: Chemokine ligand 5
CD36: Cluster of differentiation 36
CDM: Chemically-defined medium
CEBP α : CCAAT/enhance-binding protein alpha
CHOP: C/EBP homologous protein
ChoRE: Carbohydrate response element
ChREBP: Carbohydrate-responsive element-binding protein

CRISPR: Clustered regularly interspaced short palindromic repeats
CRISPRi: CRISPR interference
CXCL1: Interleukin 1
CXCL8: Interleukin 8
CYP: Cytochrome P-450
DAMP: Damage associated molecular pattern
DGAT: Diglyceride acyltransferase
DME: Drug metabolizing enzyme
DNL: De novo lipogenesis
dPBS: Dulbecco's phosphate buffered saline
ECM: Extracellular matrix
ELOVL6: Elongation of very long chain fatty acids protein 6
ENPP1: Ectonucleotide phosphodiesterase 1
ER: Endoplasmic reticulum
FABP1: Fatty acid-binding protein 1
FAC: Ferric ammonium citrate
FACS: Fluorescence-activated cell sorting
FAS: Fatty acid synthase
FATP: Long-chain fatty acid transport protein
FBS: Fetal bovine serum
FFA: Free fatty acid
FGF2: Fibroblast growth factor 2
FOXO1: Forkhead box protein O1
GADD34: Growth arrest and DNA damage inducible protein
GATA4: Transcription factor GATA-4
GCKR: glucokinase regulator
GLUT1: Glucose transporter 1
Gly: Glycine
GM-CSF: Granulocyte-macrophage colony-stimulating factor
GPAT: Glycerol-3-phosphate acyltransferase
GSK3: Glycogen synthase kinase 3
GST: Glutathione S-transferase
GWAS: Genome wide association studies
H-MRS: Proton magnetic resonance spectroscopy

HBV: Hepatitis B virus
HCC: Hepatocellular carcinoma
HCV: Hepatitis C virus
HDR: Homology directed repair
HGF: Hepatocyte growth factor
hiPSC: human induced pluripotent stem cell
HK: Hexokinase
HLC: Hepatocyte-Like Cell
HNF4a: Hepatocyte nuclear factor 4 alpha
HNF6: Hepatocyte nuclear factors
HSC: Hepatic Stellate Cell
HSD17B13: 17 β -hydroxysteroid dehydrogenase type 13
IFNL3: Interferon lambda 3
IL1 β : Interleukin 1 beta
IL6: Interleukin 6
INSIG1: Insulin induced gene 1
iPSC: Induced pluripotent stem cells
IRE1: Inositol-requiring enzyme 1
IRS-1: Insulin receptor substrate 1
JNK: c-JUN N-terminal kinases
KLF4: Kruppel-like factor 4
KO: Knock-out
LCAD: Long chain acyl dehydrogenase
LCFA: Long chain fatty acid
LDL: Low-density lipoprotein
LPAAT: Lysophosphatidic acid acyltransferase
LPC: Lysophosphatidylcholine
LPIN1: Lipin-1
LSEC: Liver Sinusoidal Endothelial Cell
LXR: Liver X receptor
MBOAT7: Membrane-bound O-acyltransferase domain containing protein 7
MCAD: Medium chain acyl dehydrogenase
MCFA: Medium chain fatty acid
MEF: Mitotically-inactivated mouse embryonic fibroblasts

MERTK: Proto-oncogene tyrosine-protein kinase MER
MGAT: Monoglyceride acyltransferase
MLX: Max-like protein X
MMP2: Matrix metalloproteinase-2
MnSOD: Superoxide dismutase
MRI: Magnetic resonance imaging
MUFA: Monounsaturated fatty acid
NAFLD: Non-alcoholic fatty liver disease
NASH: Non-alcoholic steatohepatitis
NF κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ: Nonhomologous end joining
OA: Oleic Acid
OCT4: Octamer-binding transcription factor 4
OSM: Oncostatin M
PA: Palmitic Acid
PAM: Protospacer adjacent motif
PBGD: Porphobilinogen deaminase
PCA: Principle component analysis
PERK: Protein kinase R-like endoplasmic reticulum kinase
PI3K: Phosphoinositide 3-kinase
PKC: Protein Kinase C
PNPLA3: Patatin-like phospholipase domain-containing protein 3
PPAR α : Peroxisome proliferator-activated receptor α
PPAR γ : Peroxisome proliferator-activated receptor γ
PPH-1: Serine/threonine-protein phosphatase
PPRE: Peroxisome proliferator response element
PROX1: Prospero homeobox protein 1
PUFA: Poly-unsaturated fatty acid
PUMA: p53 upregulated modulator of apoptosis
PVA: Polyvinyl alcohol
PXR: Pregnane X Receptor
ROS: Reactive oxygen species
RXR: Retinoid X receptor
S1P: Site-1 protease

S2P: Site-2 protease
SCAD: Short chain acyl dehydrogenase
SCAP: SREBP cleavage activating protein
SCD-1: Stearoyl-CoA desaturase-1
SCFA: Short chain fatty acid
Ser: Serine
SFA: Saturated fatty acid
sgRNA: Single guide RNA
SLC: Solute carrier family
SNP: Single nucleotide polymorphism
SOX2: SRY-box 2
SRE: Sterol response element
SREBP1c: Sterol regulatory element-binding protein 1c
ssODN: single-stranded donor oligonucleotides
SULT: Sulfotransferase
TALEN: Transcription activator-like effector nuclease
TCA: Tricarboxylic acid cycle
TG: Triglyceride
TGF β : Transforming growth factor β
TIMP1: TIMP metalloproteinase inhibitor 1
TIMP2: TIMP metalloproteinase inhibitor 2
TM6SF2: Transmembrane 6 superfamily 2
TNF α : Tumor necrosis factor α
TRIB1: Tribbles homolog 1
TTR: Transthyretin
UBC: Ubiquitin
UC: Untargeted Control
UCP2: Mitochondrial uncoupling protein 2
UGT: Uridine-diphospho-glucuronosyltransferase
VLCFA: Very long chain fatty acid
VLDL: Very low-density lipoprotein
ZFN: Zinc Finger Nuclease