Hfe-associated Steatohepatitis: Expression Profiling and Identifying the Molecular Basis of Liver Injury

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Master of Science (Class I Honours)

A thesis submitted for the degree of Doctor of Philosophy at

The University of Queensland in 2015

School of Medicine
Abstract

Non-alcoholic fatty liver disease (NAFLD) is an increasingly common clinical diagnosis and is predicted to become the leading indication for liver transplantation within a decade. Non-alcoholic steatohepatitis (NASH) is the progressive form of NAFLD with a fibro-inflammatory phenotype and can lead to liver cirrhosis. Although the factors responsible for progression from simple steatosis to NASH are unknown, there has been a particular interest in the role of iron-induced oxidative stress in this transition. The HFE protein plays a vital role in maintaining systemic iron homeostasis and a gene mutation in HFE is causative of haemochromatosis. In HFE-haemochromatosis, steatosis is associated with increased liver fibrosis. In keeping with these results, research from our laboratory has previously shown altered lipid metabolism and greater severity of injury in Hfe−/− mice fed a high calorie diet (HCD), which represents a western diet.

The primary aim of this project was to enhance the understanding of disease progression in Hfe-associated steatohepatitis through the identification and characterisation of genes that are differentially expressed. A gene expression profile was generated by high throughput sequencing of messenger RNA isolated from livers of Hfe−/− mice fed a chow diet and those fed a HCD. Subsequent bioinformatics analysis revealed a list of genes that were significantly altered in response to HCD-induced steatohepatitis. Among the genes that were upregulated are lipid droplet proteins, Perilipin 2 (Plin2) and Cell death inducing DFFA-like effector c (Cidec) which have been previously associated with the development of liver steatosis. Glycosylphosphatidylinositol phospholipase D1 (Gpld1), a high-density lipoprotein, was decreased in NASH livers and was the focus of this study because of the contrary upregulation observed in patients with NAFLD. Arylsulfatase G (Arsg) and Interferon, alpha-inducible protein 27 like 2B (Ifi27l2b) were identified as genes without a previously recognised role in the development of liver injury or fat accumulation and the work in this thesis has primarily focussed on elucidating the underlying roles of these genes in liver injury.

To further investigate these genes an in vitro model of hepatocyte fat and iron loading was developed. This model showed gene expression which indicated increased, mitochondrial β-oxidation and reduced fatty acid storage in cells with concomitant free fatty acid (FFA) and iron loading. These changes were also associated with an increase in the pro-inflammatory cytokine, Ccl5, indicative of a more severe injury phenotype with co-administration of FFA and iron. This model was also used to investigate hepcidin (Hamp1) expression, a key regulator
of systemic iron homeostasis, in the setting of lipid accumulation. Despite iron loading, mice fed a HCD had markedly reduced expression of Hamp1. BMP (bone morphogenetic protein) - SMAD signalling is known to be important in hepcidin induction therefore it was hypothesised that BMP signalling is altered in a fatty acid-rich environment. Consistent with this hypothesis, this study found reduced expression of BMP6 target genes when stimulated with exogenous BMP6 in cells treated with free fatty acids (FFA). This blunted response to BMP6 stimulation was due to the reduced activation of SMAD1/5/8 which is an essential component of the BMP-SMAD signalling cascade.

In this thesis, Gpld1 expression was consistently reduced in AML12 hepatocytes, with all external stimuli, FFA and iron, insulin and inflammation. This downregulation was similar to its expression in rodent NASH livers from transcriptomics analysis and suggests that Gpld1 may influence the extent of injury in iron related steatohepatitis. To the best of my knowledge this is the first study to describe a role for Arsg in response to lipid droplet accumulation and inflammation in hepatocytes and it was hypothesised that the reduced expression of Arsg, which causes lysosomal storage pathology in the brains, may indicate the role for lysosomal pathology in the development of steatohepatitis. Lastly Ifi27l2b, contrary to its described role as an interferon stimulated gene, did not cause increased apoptosis and its expression was positively correlated with protein kinase B (pAKT), a crucial protein in insulin signalling.

Kupffer cell iron loading is a common occurrence in NASH therefore the expression of ARSG, GPLD1 and IFI27L2B in iron-loaded and LPS-activated macrophages was also examined in this study. Iron administration did not result in activation of an inflammatory response in the macrophages and also did not alter the expression of ARSG, GPLD1 and IFI27L2B. However, expression of all three proteins was reduced in LPS-activated macrophages. This warrants further analysis of these genes in macrophages and the subsequent effects on hepatocyte phenotype.

In summary, studies in this thesis have identified 3 genes involved in NASH pathogenesis and have outlined their expression with a variety of external stimuli associated with the development of NASH. These studies lay the foundation for future work in this area with a particular interest in the co-administration of FFA and iron in driving changes in gene expression.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Publications:


Published abstracts:
(* denotes poster presentation, underline denotes oral presentation)


mouse model of steatohepatitis. Hepatology International, 8(suppl 1):S327. [Poster of Distinction]


6) Hepatic microRNA expression is altered in a murine model of iron and fat co-mediated liver injury. CJ McDonald, Y Yuan, N Santrampurwala, DHG Crawford, VN Subramaniam Journal of Gastroenterology and Hepatology (2014) 29 (suppl.2)


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Santrampurwala, L Britton, CHC Dejong, A Cohen, DHG Crawford, J Fawcett
Journal of Gastroenterology and Hepatology (2015)

Abstracts presented at learned societies:

Australian Liver Association workshop, Gold Coast, Queensland, June 2013
Santrampurwala N1,2, Bridle KR1,2, Heritage ML1,2, Jaskowski LA1,2, Wilkinson AS1,2, Subramaniam VN1,2,3, Crawford DHG1,2. Identification of differentially expressed genes by RNA-seq in liver of Hfe−/− mice fed a high calorie diet

School of Biomedical Sciences Post graduate symposium, Brisbane, October 2013
N Santrampurwala*, KR Bridle 1,2, ML Heritage 1,2, LA Jaskowski 1,2, AS Wilkinson 1,2, VN Subramaniam 1,2,3, DHG Crawford 1,2 Hfe-associated steatohepatitis: Expression profiling and identifying the molecular basis of liver injury

Australian Liver Association workshop, Bowral, New South Wales, May 2015
Blunted BMP6 signalling: A potential mechanism for iron loading in NAFLD N Santrampurwala, KR Bridle, J Reiling, LJ Britton, LA Jaskowski, VN Subramaniam, DHG Crawford [Early-career research award - Runner up]

American Association for the Study of Liver Diseases, San Francisco, November 2015
Publications included in this thesis

None

Contributions by others to the thesis

- **Conception and design of project**
  Prof. Darrell Crawford, Prof. Nathan Subramaniam and Dr. Kim Bridle contributed to the overall design of the project.

- **Assistance with conduct of Experiments**
  Lesley-Ann Jaskowski performed experiments including quantitative real-time PCR, western blot and immunohistochemistry. Dr Cameron McDonald assisted with some aspects of mRNA-seq and bioinformatics analysis.

- **Interpretation and troubleshooting**
  Prof. Darrell Crawford, Prof Nathan Subramaniam, Dr. Kim Bridle, Dr Jason Steel, Dr Diana Ross, Lesley-Ann Jaskowski, Dr Laurence Britton and Dr Janske Reiling significantly contributed to troubleshooting, experimental design and data interpretation. Statistical advice was provided by Dr Leesa Wockner.

- **Critical Revision of the Thesis**
  Prof. Darrell Crawford, Prof Nathan Subramaniam and Dr Kim Bridle critically revised the thesis.
Statement of parts of the thesis submitted to qualify for the award of another degree

None
Acknowledgements

First and foremost I would like to thanks my supervisors, Professor Darrell Crawford, Professor Nathan Subramaniam and Dr Kim Bridle for the opportunity to undertake my PhD under their guidance and supervision. I would like to especially thank Dr Kim Bridle who provided advice on all matters related to the lab and experimental design and also for, in the best way possible, keeping me on track to completing this thesis. I am very grateful to her for her encouragement and constant support.

I would also like to thank my colleagues from the Liver Research Centre and the Gallipoli Medical Research Foundation laboratories – Lesley Ann-Jaskowski, Janske Reiling, Laurence Britton, Erika de Guzman and Terrence Tan. Their advice in matters relating to my PhD and otherwise has provided encouragement, inspiration and entertainment towards completing this thesis. I would like to specially acknowledge the help provided by Lesley-Ann Jaskowski. Her assistance in completion of experiments in the last couple months of my PhD, where time was of utmost importance, was very helpful. Without her assistance the submission of this thesis would have been near impossible.

I am very grateful for the advice and encouragement provided by my review committee, Prof Jon Whitehead, Dr Paul Clark and Dr Michelle Mellino. I would like to thank them for critically reviewing my documents and providing feedback at yearly milestones. I would also like to thank Dr Fang Liao, Dr Jason Steel, Dr Antje Blumenthal and Prof Jon Whitehead for providing me with reagents which enabled me to perform experiments and Dr Leesa Wockner for her advice on statistical analysis.

I am also very grateful to the scholarship support provided by the Gallipoli Medical Research Foundation without which the undertaking of the PhD would not have been possible.

Last but not the least, I would like to thank my family and friends for their endless encouragement.
Keywords
Hfe<sup>−/−</sup> mice, high calorie diet, transcriptomics, NAFLD, AML12, free fatty acids

Australian and New Zealand Standard Research Classifications (ANZSRC)
ANZSRC code: 110307 – Gastroenterology and Hepatology – 100%

Fields of Research (FoR) Classification
FoR code: 1103 – Clinical Science – 100%
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AAV  adeno-associated virus
Acc  acetyl coA carboxylase
Adipo1 adiponectin receptor 1
Adipo2 adiponectin receptor 2
ALD  alcoholic liver disease
Aldh1l1 aldehyde dehydrogenase 1 family, member L1
Aldh3a2 aldehyde dehydrogenase 3 family, member A2
ALT  alanine aminotransferase
Amp-R ampicillin resistance
AMPK 5’ adenosine monophosphate-activated protein kinase
ANOVA analysis of variance
Arsg arylsulfatase G
ASH  alcoholic steatohepatitis
Atoh8 atonal homolog 8
αSMA alpha-smooth muscle actin
Bax bcl2-like protein 4
BMP  bone morphogenetic protein
BMPR bone morphogenetic protein receptor
bp  base pair
Btf3 basic transcription factor-3
B2M β-2 microglobulin
CCL5 chemokine (C-C motif) ligand 5
C282Y cysteine-to-tyrosine substitution mutation at amino acid 282
cDNA complimentary DNA
CEBPα CCAAT-enhancer binding protein alpha
CD36 fatty acyl translocase
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ChREBP</td>
<td>carbohydrate response element binding protein</td>
</tr>
<tr>
<td>Cidec</td>
<td>cell death-inducing DFFA-like effector C</td>
</tr>
<tr>
<td>CLD</td>
<td>chronic liver disease</td>
</tr>
<tr>
<td>Col1a1</td>
<td>collagen 1A1</td>
</tr>
<tr>
<td>Col3a1</td>
<td>collagen 3A1</td>
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<td>Col4a1</td>
<td>collagen 4A1</td>
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<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>Cpt1a</td>
<td>carnitine palmitoyl transferase 1A</td>
</tr>
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<td>cytochrome P450 2e1</td>
</tr>
<tr>
<td>Cox4</td>
<td>cytochrome c oxidase 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-diaminobenzidine</td>
</tr>
<tr>
<td>DcytB</td>
<td>duodenal cytochrome b</td>
</tr>
<tr>
<td>DEHP</td>
<td>di(2-ethylhexly) phthalate</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
</tr>
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<td>DIOS</td>
<td>dysmetabolic iron overload syndrome</td>
</tr>
<tr>
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<td>deionized water</td>
</tr>
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<td>divalent metal transporter 1</td>
</tr>
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<td>DNL</td>
<td>de novo lipogenesis</td>
</tr>
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<td>deoxy ribose nucleic acid</td>
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<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ERFE</td>
<td>erythroferrone</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>extracellular signal-regulated kinase ½</td>
</tr>
<tr>
<td>EtOH</td>
<td>ethanol</td>
</tr>
<tr>
<td>Fasn</td>
<td>fatty acid synthetase</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDR</td>
<td>false discovery rate</td>
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<tr>
<td>FFA</td>
<td>free fatty acid</td>
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<td>FASN</td>
<td>fatty acid synthase</td>
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<td>growth differentiation factor 15</td>
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<td>glucose transporter 4</td>
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<td>GO</td>
<td>gene ontology</td>
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<tr>
<td>Gpld1</td>
<td>glycosylphosphatidylinositol phospholipase D1</td>
</tr>
<tr>
<td>Gp130</td>
<td>glycoprotein 130</td>
</tr>
<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>reduced glutathione</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
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<tr>
<td>Gstp1</td>
<td>glutathione-s-transferase p 1</td>
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<tr>
<td>Hamp</td>
<td>hepcidin</td>
</tr>
<tr>
<td>HC</td>
<td>hepatocellular</td>
</tr>
<tr>
<td>HCC</td>
<td>hepatocellular carcinoma</td>
</tr>
<tr>
<td>HCD</td>
<td>high calorie diet</td>
</tr>
<tr>
<td>HCL</td>
<td>hydrogen chloride</td>
</tr>
<tr>
<td>HCV</td>
<td>hepatitis C virus</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HFE</td>
<td>haemochromatosis gene</td>
</tr>
<tr>
<td>Abbreviation (Gene, Compound)</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>-------------</td>
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<tr>
<td>Hfe&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Hfe-gene knockout</td>
</tr>
<tr>
<td>HIC</td>
<td>hepatic iron concentration</td>
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<tr>
<td>H&amp;E</td>
<td>haematoxylin and eosin</td>
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<td>HH</td>
<td>hereditary haemochromatosis</td>
</tr>
<tr>
<td>HIF</td>
<td>hypoxia inducible factor</td>
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<tr>
<td>HJV</td>
<td>haemouvelin</td>
</tr>
<tr>
<td>HS</td>
<td>heparan sulphate</td>
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<tr>
<td>Hsd3b5</td>
<td>hydroxy-delta-5-steroid dehydrogenase, 3 beta</td>
</tr>
<tr>
<td>Hsd17b13</td>
<td>hydroxy-delta-17-steroid dehydrogenase, 13 beta</td>
</tr>
<tr>
<td>HSPG</td>
<td>heparan sulphate proteoglycan</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O&lt;sub&gt;2&lt;/sub&gt;</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>H63D</td>
<td>histidine-to-aspartate substitution mutation at amino acid 63</td>
</tr>
<tr>
<td>Ifi27l2b</td>
<td>interferon alpha-inducible protein 27 like 2B</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IR-HIO</td>
<td>insulin resistance associated hepatic iron overload</td>
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<td>IR</td>
<td>insulin resistance</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>IRS1</td>
<td>insulin receptor substrate 1</td>
</tr>
<tr>
<td>ISP</td>
<td>ion sphere particle</td>
</tr>
<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>Kbp</td>
<td>kilo base pair</td>
</tr>
<tr>
<td>Kcl</td>
<td>potassium chloride</td>
</tr>
<tr>
<td>KO</td>
<td>knock out</td>
</tr>
<tr>
<td>LB</td>
<td>luria broth</td>
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<tr>
<td>LFABP</td>
<td>liver fatty acid binding protein</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LTx</td>
<td>liver transplantation</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Min</td>
<td>minute</td>
</tr>
<tr>
<td>Mm10</td>
<td><em>Mus musculus</em> genome annotation 10</td>
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<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<td>mRNA-seq</td>
<td>messenger RNA sequencing</td>
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<td>MUFA</td>
<td>monounsaturated fatty acid</td>
</tr>
<tr>
<td>MUP</td>
<td>major urinary protein</td>
</tr>
<tr>
<td>NAD</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
</tr>
<tr>
<td>NAFLD</td>
<td>nonalcoholic fatty liver disease</td>
</tr>
<tr>
<td>NAS</td>
<td>NAFLD activity score</td>
</tr>
<tr>
<td>NASH</td>
<td>nonalcoholic steatohepatitis</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>NFκB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>nM</td>
<td>nano molar</td>
</tr>
<tr>
<td>NT</td>
<td>non-targeting</td>
</tr>
<tr>
<td>Na₄P₂O₇</td>
<td>sodium pyrophosphate</td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>NFκβ</td>
<td>nuclear factor- kappa beta</td>
</tr>
<tr>
<td>Nrf1</td>
<td>nuclear respiratory factor-1</td>
</tr>
<tr>
<td>NTBI</td>
<td>non-transferrin bound iron</td>
</tr>
<tr>
<td>Nrf1</td>
<td>nuclear respiratory factor 1</td>
</tr>
<tr>
<td>OH</td>
<td>hydroxyl radical</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>Orm</td>
<td>orosomucoid</td>
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<tr>
<td>O$_2^-$</td>
<td>superoxide</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PGM</td>
<td>personal genome machine</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>Plin2</td>
<td>adipose differentiation related protein</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PrP</td>
<td>prion protein</td>
</tr>
<tr>
<td>PSC</td>
<td>primary sclerosing cholangitis</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
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<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>Q3</td>
<td>quartile 3</td>
</tr>
<tr>
<td>RE</td>
<td>restriction endonuclease</td>
</tr>
<tr>
<td>RES</td>
<td>reticuloendothelial system</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>RPM</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>RPKM</td>
<td>reads per kilobase of exon model per million mapped reads</td>
</tr>
<tr>
<td>R-SMAD</td>
<td>receptor SMAD</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>real time-quantitative polymerase chain reaction</td>
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<tr>
<td>Saa</td>
<td>serum amyloid A</td>
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<tr>
<td>Sbp2</td>
<td>selenium binding protein 2</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Scd1</td>
<td>stearoyl coA desaturase 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
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<tr>
<td>Scnn1a</td>
<td>sodium channel, non-voltage gated 1 alpha subunit</td>
</tr>
<tr>
<td>Slco1a1</td>
<td>solute carrier organic anion transporter family, member 1a1</td>
</tr>
<tr>
<td>Slco2a1</td>
<td>solute carrier organic anion transporter family, member 2a1</td>
</tr>
<tr>
<td>Smad</td>
<td>mothers against decapentaplegic</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Srebp1c</td>
<td>sterol regulatory element binding protein 1c</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>Tf</td>
<td>transferrin</td>
</tr>
<tr>
<td>TfR1</td>
<td>transferrin receptor 1</td>
</tr>
<tr>
<td>TfR2</td>
<td>transferrin receptor 2</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TMAP</td>
<td>torrent mapping alignment program</td>
</tr>
<tr>
<td>Tor1b</td>
<td>torsin 1b</td>
</tr>
<tr>
<td>Tnfa</td>
<td>tumour necrosis factor-α</td>
</tr>
<tr>
<td>Tgfβ</td>
<td>transforming growth factor-β1</td>
</tr>
<tr>
<td>TWSG1</td>
<td>twisted gastrulation protein homolog 1</td>
</tr>
<tr>
<td>VCAM1</td>
<td>vascular adhesion molecule 1</td>
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Chapter 1  Literature Review
1.1 General introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease in the developed world and is caused by the accumulation and deposition of fat in the liver in the absence of excessive alcohol consumption (1). NAFLD encompasses a spectrum of liver disorders from simple steatosis to non-alcoholic steatohepatitis (NASH) and is a disease of growing prevalence and increased global burden. NAFLD is present in 20-30 % of adults in the developed world and is proposed to become the leading cause of liver transplantation in approximately a decade (2-4). NASH is the progressive form of NAFLD comprising steatosis associated with hepatic necroinflammation, ballooning, and varying degrees of fibrosis (5). As is, simple steatosis is a benign condition but in a subset of patients may progress to NASH and potentially develop into cirrhosis, end stage liver disease and hepatocellular carcinoma. It is not well understood why some patients develop a fibro-inflammatory phenotype but there has been a particular interest in the role of iron induced oxidative stress in this transition.

In keeping with this, mutations in the HFE gene – a regulator of systemic iron homeostasis and the molecular basis of Type 1 Hereditary Haemochromatosis – have been observed in patients with NASH with excess hepatic iron (6-8). Steatosis in patients with the C282Y HFE mutation has also been observed and was found to be an independent risk factor for the progression of fibrosis (9). Our research group has observed similar evidence in Hfe−/− mice fed a high calorie diet (HCD). This study showed a significant increase in serum alanine amino transferase (ALT), a marker of liver injury, and increased hepatic inflammatory and fibrogenic gene expression in Hfe−/− mice fed a HCD which develop steatohepatitis and early fibrosis (10). The mechanisms underlying the development of more severe injury are however not fully understood and require further investigation.

Research conducted in the field has been largely limited to the study of either Hfe−/− or fat accumulation and this project has aimed to investigate the co-toxicity of HFE-related iron overload and fat accumulation in the development of steatohepatitis.

1.2 Iron biology and homeostasis

Iron plays a crucial role in vital biochemical activities such as oxygen sensing, electron transfer and catalysis. A significant portion of the cellular iron is found in two major classes of proteins – haemoproteins (haeme centres) and those with iron-sulphur clusters (Fe-S) – which are involved in enzyme catalysis, electron transport and oxygen transport (11, 12). Iron is one
element which can exist in two oxidation states – ferrous (Fe\(^{2+}\)) and ferric (Fe\(^{3+}\)) – making it capable of a diverse range of biological functions. It is this redox potential of iron that also makes it especially toxic when in abundance. Under aerobic conditions, iron can readily catalyse and form toxic radicals via the Fenton and Haber-Weiss chemical reaction (Fig 1.1) to form hydroxyl and superoxide radicals, commonly called reactive oxygen species (ROS) (13).

\[
\text{Fenton reaction} \\
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \cdot\text{OH} \\
\text{OH}^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2^- + \text{H}^+ + \text{H}_2\text{O}
\]

\[
\text{Haber-Weiss reaction} \\
\text{O}_2^- + \text{H}_2\text{O}_2 \xrightarrow{\text{Fe}^{3+/2+}} \text{OH}^- + \cdot\text{OH}^- + \text{O}_2
\]

**Fig 1.1: Fenton and Haber-Weiss reaction.** The Fenton reaction is the reaction of ferrous iron (Fe\(^{2+}\)) and hydrogen peroxide (H\(_2\)O\(_2\)) which produces ferric iron (Fe\(^{3+}\)) and hydroxyl radicals. The hydroxyl radical then reacts with H\(_2\)O\(_2\) to produce superoxide (O\(_2^-\)). Then superoxide reacts again with H\(_2\)O\(_2\) and hydroxyl radical and hydroxyl anion (-OH) are formed. This reaction is known as the Haber-Weiss Reaction.

The generation and accumulation of ROS beyond the systemic antioxidant capacity can cause severe inflammation and tissue degeneration. Free radicals are highly reactive species and can cause oxidation of fatty acids, DNA, proteins and membrane phospholipids (14). Hence iron is usually bound to proteins such as transferrin and ferritin to avoid the deleterious effects of ROS and body iron homeostasis is tightly regulated to maintain the iron levels within a physiologically optimum range.

Dietary iron is the only source of iron and is absorbed by intestinal enterocytes and is stored in the liver from where it is mobilised in times of need. The human body requires 1-2 mg of iron per day to replace lost iron (15) and is distributed to various cells and tissues and is depicted in Fig 1.2. Systemic iron homeostasis is maintained by affecting iron absorption and the mobilisation of iron stores in times of erythropoietic demand. There is no known regulated mechanism for iron export from the body hence intestinal absorption by duodenal enterocytes is under tight regulation. Dietary iron is predominantly available as ferric (Fe\(^{3+}\)) iron which is reduced to ferrous (Fe\(^{2+}\)) iron by duodenal cytochrome B (DCYTb) and enters the enterocyte
via the divalent metal transporter 1 (DMT1). The iron is either stored in the enterocytes as ferritin or exported via the only known iron export protein ferroportin (FPN), into the portal circulation (Fig 1.3) and the circulating iron is bound to plasma transferrin which is distributed within the body for cellular utilisation and storage (16).

![Diagram of iron traffic in the body](image)

**Fig 1.2: Overview of body iron requirements.** This schema depicts the major pathways of iron traffic in the body with the approximate daily utilization stated. 1-2 mg of iron is absorbed in the duodenum and enters the circulation as transferrin bound iron. Bound to transferrin (Tf), iron circulates through the various tissues and cells or is stored in the liver. Effective communication between these organ systems is essential to maintain effective absorption, utilisation and mobilisation of stored iron. Iron excretion from the body is not regulated and can occur via blood loss and shedding of cells from the skin and mucosal lining. This figure has been reproduced with permission from *Cell, Volume 117, 2004* (17).

### 1.3 Hepcidin: The key regulator of systemic iron homeostasis

Intestinal iron absorption is influenced by body iron requirements and is regulated primarily by the basolateral iron exporter FPN via a liver derived hormone: hepcidin. Hepcidin is a small
peptide hormone which is predominantly synthesized in the liver and is a negative regulator of systemic iron homeostasis (18-20).

Hepcidin binds to FPN leading to its internalisation and degradation hence reducing iron efflux from the duodenal enterocyte into the blood stream in conditions of iron overload (21, 22). FPN is also expressed on hepatocytes and reticuloendothelial macrophages and iron retention in these cells is increased with increased hepcidin expression (low abundance of basolateral transporter FPN). Evidence for the described role of hepcidin as a regulator of systemic iron homeostasis comes from murine models of targeted hepcidin deletion having severe iron overload in the liver and other organs (23-25).

1.3.1 Regulation of Hepcidin

Hepcidin expression is regulated by erythropoietic demand, hypoxia, inflammation and iron status (Fig 1.3). Erythropoiesis, the production of red blood cells, increases the demand for iron and increased erythropoiesis reduces hepcidin expression. This is potentially regulated via growth differentiation factor 15 (GDF-15) and twisted gastrulation protein homolog 1 (TWSG-1), proteins which are secreted by the bone marrow with increased erythropoietic demand (26). A recently identified erythroid regulator, erythroferrone (ERFE) has also suppressed hepcidin expression on stimulation of erythropoiesis (27). Hypoxia (low oxygen tension) regulates hepcidin expression by regulating the availability of haemojuvelin (HJV:BMP6 co-receptor) through the activity of hypoxia inducible factors (HIFs) and membrane proteases which cleave HJV, reducing BMP mediated signalling and hence reduced hepcidin expression (26, 28).

Hepcidin synthesis on the other hand, is positively regulated by infection and inflammation (29). The increase in hepcidin expression is thought to be mediated by the pro-inflammatory cytokine, interleukin 6 (IL6) through the Janus kinase-signal transducer and activation of transcription (JAK-STAT) signalling pathway (30). IL6 binds to the IL6 receptor α and glycoprotein 130 (Gp130) complexes on the hepatocyte cell surface which activates JAK. Activated JAK subsequently phosphorylates signal transducer and activator of transcription 3 (STAT3). STAT3 then translocates to the nucleus, binds to its specific promoter and enhances HAMP transcription (26, 28, 31, 32). IL6 treatment of humans, mice, primary hepatocytes and hepatocyte cell lines all demonstrate a similar induction of hepcidin (33).

Iron status is also known to positively regulate hepcidin expression by the following proposed mechanisms. Firstly, liver iron stores can regulate hepcidin expression through the extracellular
signalling molecule bone morphogenetic protein 6 (BMP6) which interacts with the BMP receptor (BMPR) and HJV to initiate signalling via the mothers against decapentaplegic (SMAD) signalling pathway (28, 32). The crucial role of the BMP-SMAD signalling pathway in regulating hepcidin was demonstrated by the liver specific deletion of SMAD4 which resulted in very low levels of hepcidin and development of an iron overload phenotype (34). In another study, SMAD4−/− mice were also shown to be unable to regulate hepcidin expression in response to inflammation and iron overload (35). Secondly, transferrin-bound circulating iron can bind to transferrin receptor 1 (TfR1) which interacts with the haemochromatosis protein, HFE and signals transcriptional activation of hepcidin through mitogen-activated protein kinase or SMAD signalling pathways (28, 36).

To summarise, increased erythropoietic activity and hypoxia reduce hepcidin expression while increased inflammation and high serum iron concentration increase hepcidin expression to modulate iron absorption from the duodenum (Fig 1.3). The exact molecular mechanisms are yet to be identified, but there is sufficient evidence to support the proposed model that increased hepcidin transcription and reduced abundance of basolateral transporter FPN leads to reduced iron efflux from the duodenum. Conversely, reduced hepcidin expression allows increased efflux of iron from the duodenum into the blood stream. These signalling pathways are tightly regulated and irregularities of these pathways can cause severe pathologies of iron loading and deficiency.
Fig 1.3: Regulation of iron absorption by Hepcidin. Ferrous iron enters the enterocytes via DMT1 and under high iron or inflammatory conditions hepcidin secreted by the liver binds to FPN on the duodenal enterocytes and macrophages to regulate iron efflux. Hepcidin expression is regulated by iron status through 1) HFE and TfR1 interaction and 2) BMP/BMPR/HJV interaction by inducing SMAD signalling and inducing hepcidin expression. Inflammation via IL6 and STAT3 signalling also drives hepcidin expression. Divalent metal transporter 1 (DMT1), ferroportin (FPN), transferrin receptor 1 (TfR1), bone morphogenetic protein (BMP), BMP receptor (BMPR), haemojuvelin (HJV), interleukin-6 (IL6). This figure has been reproduced with permission from *Crit Rev Clin Lab Sci, Volume 44, 2007* (36).

### 1.4 HFE haemochromatosis: An iron loading disorder

Iron overload disorders are characterised by increased plasma transferrin saturation and elevated serum ferritin, and can occur via defects in the hepcidin-ferroportin axis, impaired iron transport or ineffective erythropoiesis. Hereditary haemochromatosis is an iron loading disorder which is characterised by increased iron absorption from the gastrointestinal tract leading to accumulation of iron in tissue parenchyma which may lead to consequent tissue pathology and organ damage (37). Hereditary haemochromatosis is an autosomal recessive disorder which is caused by a mutation in the *HFE* gene (38). The HFE protein is a member of the major histocompatibility complex (MHC) class-1 like family (39). While the HFE protein
has structural similarities to the MHC class 1 protein family which mediates signalling to cytotoxic T-cells, it does not have any known role in mounting an immune response. On the contrary, HFE has a role in regulation of body iron homeostasis. It interacts with TfR1 to initiate a signalling cascade which drives hepcidin expression (40) and consequently, mutations in the HFE gene cause the most prevalent primary iron loading disorder: HFE-haemochromatosis (HH) or Type 1 haemochromatosis (39). HH is characterised by inappropriately low levels of hepcidin expression (41, 42), increased duodenal iron absorption in spite of adequate body iron stores, high transferrin saturation and iron deposition in various organs which may lead to fibrosis and ultimately hepatic cirrhosis (43).

The mutation in the HFE gene, the substitution of tyrosine for cysteine at amino acid 282 (C282Y), is the most common in Caucasian populations with approximately 80-90 % of HH patients homozygous for this mutation (44, 45). In other ethnicities this mutation is less common. The C282Y substitution mutation disrupts a disulphide bridge and prevents the association of HFE and β2-microglobulin which is a necessary step in processing of HFE without which it undergoes proteasomal degradation and reduced cell surface expression (46, 47). Another more common mutation for HH, the substitution of aspartate for histidine at amino acid 63 (H63D) was also identified. This mutation sometimes results in increased transferrin saturation but without clinically significant iron loading (48).

Despite excess iron stores, HFE-haemochromatosis patients and Hfe knockout mice have decreased expression of hepcidin (42, 49). A model for the role of HFE in hepcidin regulation has been proposed which involves TfR1, transferrin receptor 2 (TfR2), HJV and signalling molecules BMP and SMAD proteins (Fig 1.4). In low iron conditions, HFE remains bound to TfR1 and hence hepcidin signalling is switched off, but in high iron conditions HFE forms a complex with BMP, HJV and TfR2 to turn on hepcidin expression and reduce systemic iron absorption. The dysregulation of this mechanism is potentially the cause for low hepcidin expression in HH. Decreased hepcidin expression causes ferroportin mediated efflux of iron from enterocytes allowing absorption of iron in spite of high body iron stores (50).
Fig 1.4: A model for HFE mediated regulation of hepcidin. A) At low plasma iron concentrations HFE remains bound to TfR1 and hepcidin transcription is suppressed. B) In high iron conditions HFE forms a complex with HJV, BMP and TfR2 and turns on hepcidin expression via the SMAD signalling pathway. Transferrin receptor 1 (TfR1), haemojuvelin (HJV), bone morphogenetic protein (BMP), transferrin receptor 2 (TfR2). This figure has been reproduced with permission from WJG, Volume 14, 2008 (50).

1.4.1 Hfe deficiency and hepcidin regulation

Hepcidin expression is directly regulated by body iron status through mechanisms which involve BMPs and are regulated by the SMAD signalling intermediaries. BMPs are growth factors which belong to the transforming growth factor-beta (TGFβ) superfamily of regulatory proteins. BMPs are very versatile and while they were initially characterised as proteins with a role in bone and cartilage formation, they also have roles in cellular differentiation, proliferation and survival, vascular homeostasis and iron metabolism (51). In vitro studies have shown that several BMPs can induce hepcidin expression in hepatic cells and administration of BMP2 in mice causes a substantial increase of hepcidin (52). Of the several BMPs with a capacity to induce hepcidin expression, BMP6 is most potent. Consistent with this, knockout of BMP6 results in low hepcidin expression and a severe iron loading phenotype (34, 53). BMP6 levels are increased in response to high body iron stores (54) and Bmp6 knockout mice have almost undetectable levels of hepcidin and develop systemic iron overload (55, 56).
BMP receptors (BMPR) on the hepatocyte surface consist of serine-threonine kinases type I and type II and recognise the BMP ligands. Upon BMP binding, the type II receptor activates the type I receptor kinase which releases the inhibitor FK506-binding protein 12 (FKBP12), leading to the recruitment and subsequent phosphorylation of its substrate, an intracellular receptor SMAD (R-SMAD), SMADs 1, 5 and 8 (SMAD 1/5/8) (57). The phosphorylated SMAD1/5/8 then binds to SMAD4 to form a complex which translocates to the nucleus and binds to its specific BMP responsive element to affect transcription of its target genes such as hepcidin (58, 59) (Fig 1.5).

HFE is also involved in the regulation of hepcidin expression via the BMP-SMAD signalling pathway (60). It forms a complex with TfR1 on the surface of hepatocytes and this interaction is disrupted by circulating transferrin bound iron which has a higher affinity for TfR1 (61). Upon dissociation from TfR1, HFE complexes with TfR2 and the BMP co-receptor: HJV which initiates signals to activate the BMPR which in turn activates the signalling to its downstream target genes (61, 62). An alternative mechanism for hepcidin signalling in the absence of the HFE/TfR2 complex has also been described which suggests that HFE and TfR2 can independently regulate hepcidin expression (63, 64). The molecular pathways which signal HAMP synthesis in response to the HFE/TfR2 iron-sensing complex or conversely their independent actions are an area of intense investigation and evidence to date suggests a role for ERK1/2 and a possible interaction with the BMP/SMAD pathway (65-67) (Fig 1.5).

Consistent with this model, BMP-SMAD signalling was impaired in Hfe knockout mice (68). Hfe knockdown resulted in increased BMP6 mRNA as would be expected with an iron loading phenotype. Despite increased BMP6 expression, phosphorylation of SMAD1/5/8 was reduced as was the expression BMP6 target genes: Id1 and Hamp1 (68). Conversely, overexpression of Hfe resulted in increased phosphorylation of SMAD1/5/8 (pSMAD1/5/8), excess hepcidin and consequently an iron deficient anaemia phenotype. This increased phosphorylation was independent of BMP6 mRNA expression suggesting a role for HFE activation of the BMP-SMAD pathway down stream of BMP ligand activation (69). In the same study, administration of a high dose of exogenous BMP6 increased hepatic Hamp1 expression despite Hfe knockdown, suggesting that enhanced BMP6 expression beyond a threshold can activate downstream SMAD signalling independent of a HFE interaction.

Additionally, there is evidence for hepcidin regulation by iron status despite attenuation of SMAD1/5/8 phosphorylation in Hfe and Tfr2 knockout mice. In Hfe/Tfr2 double knockout
mice, supplementation of iron increased hepcidin expression without an increase in pSMAD1/5/8 (70).

The above evidence all suggest an important role for HFE, Tfr2 and BMP6 in iron regulation. These molecules can function independently of the others but the exact mechanisms are unknown and require further investigation.

**Fig 1.5: Overview of hepcidin signalling pathways.** Transferrin bound iron preferentially binds Tfr1, allowing the association of HFE and Tfr2. This complex then binds to HJV and BMPR I/II, activating a signalling cascade which involves phosphorylation of SMAD1/5/8, subsequent binding to SMAD4 and translocation to the nucleus where it recognises the BMP responsive element to turn ON transcription of hepcidin. HFE and TFR2 may also signal hepcidin expression independently or as a complex via the ERK signalling pathway. Transferrin receptor 1 (Tfr1), transferrin receptor 2 (Tfr2), haemojuvelin (HJV), bone morphogenetic protein receptor I and II (BMPR I/II), mothers against decapentaplegic protein family (SMAD), extracellular signal-regulated kinase (ERK). This figure has been reproduced with permission from *Front Pharmacol*, Volume 5, 2014 (60)

### 1.4.2 Prevalence and clinical penetrance of HFE haemochromatosis

Although 10-15 % of the Caucasian population are carriers of the *HFE* mutation (71) but this is not represented in the clinical presentation of this disorder since many C282Y carriers do not present with clinically significant iron overload (45, 72, 73). In a study by Adams *et al* in 2005,
76% C282Y homozygotes were undiagnosed with iron overload. Amongst the C282Y homozygotes, 88% men had > 300 μg/L serum ferritin and 57% women had > 200 μg/L serum ferritin (72). Thirteen percent of C282Y homozygotes had serum ferritin greater than 1000 μg/L, which is a threshold associated with increased risk of cirrhosis. In another study, Allen et al reported 19% of C282Y homozygotes had serum ferritin greater than 1000 μg/L (74). Of the subset of HH subjects who develop clinically significant hepatic iron concentration (HIC) only a proportion (~7%) develop cirrhosis and significant fibrosis has developed despite lower HICs (73, 75, 76). HH patients have varying susceptibilities to iron induced fibrosis and genetic and environmental factors may play a role in the varying penetrance and progression of HFE-related liver injury. In keeping with this, one study found a strong relationship between steatosis and portal fibrosis with HH (9), hence steatosis has been implicated as a co-factor in liver injury.

### 1.5 Non-alcoholic steatohepatitis (NASH)

#### 1.5.1 Pathogenesis of NASH

NAFLD is the most common chronic liver disease in western countries (77) and increasing prevalence in Asian countries (78, 79). The occurrence of obesity along with dyslipidaemia, impaired glucose tolerance and hypertension is referred to as the metabolic syndrome (80) and NAFLD is often referred to as the hepatic manifestation of the metabolic syndrome which is commonly associated with insulin resistance, oxidative stress and the dysmetabolic iron overload syndrome (DIOS). The earliest stage of NAFLD is characterised by the deposition of cytoplasmic triglycerides as macro and/or microvesicular lipid droplets. The excessive accumulation of triglycerides in the hepatocytes arises from an imbalance of free fatty acid uptake and removal. The primary source of fatty acids is lipolysis of adipose tissue (60%) and increase in de novo lipogenesis (25%) and is exacerbated by a hypercaloric diet which accounts for the remaining burden of hepatic fat loading (81). Hepatic fatty acid accumulation also results from reduced very low density lipoprotein (VLDL) export from the liver (Fig 1.6). These excess free fatty acids then undergo esterification to form triglyceride molecules.

Triglyceride accumulation and lipid droplet formation as a result of increased free fatty acids in the liver is the key element for the development of steatosis and paradoxically lipid droplet formation confers protection from liver injury. Evidence for this comes from studies which
have shown that inhibition of triglyceride synthesis improved liver steatosis but led to worse liver injury (82). The alternative to storage of the excess free fatty acids is their break down via mitochondrial β-oxidation which, when unchecked, can also act as a source of ROS and subsequent oxidative stress which promotes development of steatohepatitis (83, 84).

![Diagram of fatty acid metabolism](image.png)

**Fig 1.6: Contribution of various pools of free fatty acids in the development of non-alcoholic fatty liver disease.** Approximately 60% of the FFA pool in the liver is derived from subcutaneous fat. A hypercaloric diet and the resulting hyperinsulinaemia increase *de novo* lipogenesis (DNL) and contribute to the existing pool of free fatty acids. This increased FFA pool is concurrent with reduced mitochondrial β-oxidation hence promoting formation of lipid droplets and increasing the pool of hepatic triglycerides. VLDL export from the hepatocytes is also reduced. Carbohydrate response element-binding protein (ChREBP), chylomicron remnants (CM rem), *de novo* lipogenesis (DNL), fatty acids bound to coenzyme A (FA CoA), free fatty acids (FFA), subcutaneous (SC), sterol regulatory element-binding protein 1 (SREBP1), triglycerides (TG), very low density lipoproteins (VLDL). This figure has been reproduced with permission from *Nat Clin Pract Endocrinol Metab, Volume 2, 2006* (85).

The prognosis of NAFLD is usually benign but can be complicated by the development of NASH which can progress to cirrhosis and end-stage liver disease (86). The development of NASH has been proposed to occur via the “two hit hypothesis” where insulin resistance associated with triglyceride accumulation is the first hit followed by a second hit of oxidative stress, mitochondrial dysfunction, lipotoxicity or the release of pro-inflammatory cytokines resulting in tissue injury, steatohepatitis and fibrosis (87). In addition to the role of free fatty
acids in the development of steatohepatitis there has also been an increasing interest in the role for cholesterol accumulation in exacerbating liver injury (88, 89) and cholesterol reversal has reduced steatohepatitis pathology (90). A multiple parallel hit hypothesis has also been proposed which suggests that many insults occur simultaneously which result in fat infiltration and hepatic inflammation (91). Some possible factors implicated in the pathogenesis of NASH include inflammatory cytokines (92), dysregulated adipokine production (93), altered gut microbiota (94), endoplasmic reticulum stress (95) and endotoxemia as a result of increased gut permeability (96). Regardless of the hypothesis for the development of steatohepatitis, liver damage seems to converge on a combination of insulin resistance and fatty acid accumulation which drives cellular injury via oxidative stress, insulin resistance and inflammation (91, 97).

1.5.2 Insulin resistance and NASH

Insulin resistance is defined as the physiological state in which the cells fail to respond to normal levels of insulin and the development of NAFLD is associated with the presence of insulin resistance (97). While lipotoxicity is central to the pathogenesis of steatohepatitis, its concurrence with insulin resistance might be integral to the development of progressive liver injury. Evidence for this comes from studies in which despite the development of obesity the mice were protected from liver injury in an environment of improved insulin sensitivity (98, 99).

The key sites of insulin action are the liver, skeletal muscle and adipose tissue. Hyperinsulinaemia promotes adipose tissue lipolysis and generates the majority of the flux of FFA to the liver (84). Insulin also promotes de novo lipogenesis in the liver through the transcriptional factor sterol regulatory element-binding protein-1c (Srebp1c) which then stimulates acetyl-coA carboxylase (Acc), fatty acid synthase (Fasn) and stearoyl-coA desaturase (Scd1) (100, 101) all enzymes involved in the synthesis of free fatty acids. In an over-fed state, the increase in lipogenesis results in accumulation of malonyl co-A which inhibits carnitine palmitoyl transferase 1 (CPT1a), the shuttle of FFA into the mitochondria, and hence leads to a reduction of mitochondrial β-oxidation (Fig 1.7).

Furthermore, inflammation is proposed to be a crucial element in the development of NASH and several inflammatory cytokines can act as mediators in the development of insulin resistance (97).
Fig 1.7: Insulin resistance stimulated molecular changes leading to hepatic triglyceride accumulation. Insulin resistance is characterised by hyperinsulinaemia and increased hepatic glucose production. Insulin resistance in adipose tissue leads to HSL induced adipolysis which increases FFA flux to the liver. In the liver, hyperinsulinaemia and hyperglycaemia activates Srebp1c and Chrebp1 respectively which induce de novo lipogenesis and consequently reduce mitochondrial β-oxidation. In the setting of insulin resistance, the FFA derived from adipolysis and de novo lipogenesis are then esterified to form triglycerides. Hormone sensitive lipase (HSL), sterol regulatory element binding protein-1c (Srebp1c), carbohydrate binding-element protein 1 (Chrebp1), free fatty acids (FFA), very low density lipoprotein (VLDL). This figure has been reproduced with permission from J Clin Invest, Volume 4, 2004 (102).

1.5.3 Oxidative stress and NASH

Oxidative stress has been implicated in the development of steatohepatitis and many human and animal studies have found an association between NASH and biomarkers of oxidative stress (103-106). In a state of high energy demand, the increased FFA flux in NAFLD leads to an increase in mitochondrial β-oxidation of FFA, and hence increased electron flux through the electron transport chain, a process during which the mitochondria leak ROS mainly in the form of hydrogen peroxide (107). In order to prevent oxidative stress, an antioxidant defence system involving enzymes such as superoxide dismutase, glutathione peroxidase, catalase and thioredoxin is available and can inactivate the ROS and nullify the consequent deleterious effects (108). Additionally, in NAFLD and NASH a study has reported reduced anti-oxidant capacity which exacerbates oxidative stress (103).
In a FFA-rich environment however, mitochondrial β-oxidation is overactive to oxidise the excess FFA, hence producing excessive oxidative radicals via the electron transport chain. The mitochondrial capacity to control the oxidative balance collapses under continuous oxidative stress conditions and leads to ROS-induced lipid peroxidation of the accumulated hepatic fat which then triggers steatohepatitis development (109) (Fig 1.8). ROS also has the ability to induce the secretion of cytokines such as tumour necrosis factor gamma (TNFγ), transforming growth factor beta (TGFβ) and interleukin-8 (IL8) which can lead to collagen synthesis, neutrophil infiltration and the development of fibrosis (110, 111). Evidence from an in vitro study has also shown that ROS production as a result of fatty acid accumulation has led to disrupted lipid storage, dysregulated expression of adipocytokines and insulin resistance (112). Additionally the use of antioxidant therapy to scavenge the excess free radicals has been shown to be useful in the amelioration of steatohepatitis indicating an involvement of free radicals in the development of steatohepatitis (111). A number of studies have investigated the effects of vitamin E, a free radical scavenger, to show an improvement in histological parameters of NASH (113, 114).

Given the role of iron as a potent catalyst of ROS, oxidative stress induced by iron overload has been implicated in the progression of injury (5, 87). The saturated antioxidant defence system in a FFA rich environment also renders the liver more susceptible to iron induced oxidative injury and recent studies have elucidated a variety of roles for iron in the pathogenesis of NASH by mediating alteration of insulin signalling and lipid metabolism. These mechanisms are discussed in greater detail below.

1.6 The role of iron in NASH pathogenesis

1.6.1 Prevalence of iron in NASH

Hepatic iron accumulation is a source of oxidative stress which leads to oxidation of biomolecules and consequent hepatocyte dysfunction. The role of iron in NASH pathogenesis is debated and remains controversial (115). Iron in NAFLD patients was first reported in 1994 by Bacon et al. who detected increased serum iron and high transferrin saturation (86). The first report of increased stainable iron in hepatocytes was reported by George et al. (7) who found that the HIC correlated positively with the degree of fibrosis. Hepatic iron deposition can occur in one of three patterns: hepatocellular (HC) or parenchymal deposition only, reticuloendothelial system (RES) deposition only, or a mixed pattern of HC and RES iron deposition (116).
**Fig 1.8: Free fatty acid and oxidative stress mediated NASH pathogenesis.** Insulin resistance and increased dietary intake of saturated fatty acids leads to increased FFA flux into the hepatocytes. The increased FFA is either esterified to form protective triglyceride droplets or is catabolised via mitochondrial β-oxidation. In a disease condition however these systems are overwhelmed and results in accumulation of ROS which leads to lipid oxidation and consequent lipotoxicity and the development of steatohepatitis. Green arrow (protective mechanism), red arrow (injurious mechanisms), insulin resistance (IR), carbohydrate (CHO), saturated fatty acids (SFA), free fatty acids (FFA), reactive oxygen species (ROS). This figure has been reproduced with permission from *Int J Mol Sci, Volume 15, 2014* (117).

Recent human studies have shown an association between the presence and pattern of hepatic iron deposition and the severity of NAFLD (118, 119). RES iron deposition was associated with steatohepatitis: higher NAFLD activity scores (NAS) and elevated serum alanine aminotransferase (ALT). HC and mixed pattern of iron deposition on the other hand were associated with a milder histologic phenotype. Only RES iron deposition was associated with advanced hepatic fibrosis (119). In another study, RES iron was also shown to be more prevalent in hepatocellular carcinoma (HCC) (120). In contrast to these results however, a study by Valenti *et al.* has shown that the prevalence of fibrosis stage >1 was higher in patients with HC iron deposition (121). Results from yet another study by Maliken *et al.* showed that both HC and RES iron deposition are associated with increased oxidative stress however,
patients with RES iron deposition had induced apoptosis and an increased prevalence of NASH, while patients with HC iron deposition developed cell necrosis and did not have significant effects on NASH (122).

A number of studies have since investigated the role of hepatic iron in NAFLD and found no correlation of iron loading (irrespective of the pattern) and fibrosis (123-126). Hyperferritinemia however, has been frequently associated with NASH and the term insulin resistance-associated hepatic iron overload (IR-HIO) has been coined to describe the association between hepatic steatosis and hepatic iron overload (126-130).

1.6.2 Iron mediated pathogenesis

The role of iron overload in NAFLD is thought to be related to its ability to produce ROS. ROS initiates oxidative stress which can cause lipid peroxidation, mitochondrial dysfunction, endoplasmic reticulum stress and necroinflammation (131). In steatotic livers, the saturation of the mitochondrial electron transport chain and peroxisomal β-oxidation by excess fatty acid oxidation can lead to generation of hydrogen peroxide (132-134), and in the presence of free iron the hydrogen peroxide can be converted to toxic free radicals by the Fenton reaction and cause oxidative stress (135). Several other studies have demonstrated a link between iron induced oxidative stress and disease severity: markers of oxidative stress such as serum thioredoxin were increased in NASH (136), haeme oxygenase 1, a sensitive indicator of oxidative stress was correlated with levels of serum ferritin and lipid peroxidation (137) and 8-hydroxy-2′-deoxyguanosine, a marker of oxidative DNA adducts was positively correlated with hepatic iron score and serum ferritin levels (138). Oxidative stress also leads to lipid peroxidation and generation of malondialdehyde and 4-hydroxynonenal which can mediate the upregulation of pro-inflammatory cytokines like nuclear factor kappa-light-chain-enhancer of activated B-cells (NFκB), tumour necrosis factor-alpha (TNFα) and IL6 – thus inducing inflammation, fibrogenesis and apoptosis (105). Additionally, liver macrophages are known to accumulate iron and macrophage iron status has been shown to affect their inflammatory response (139).

Increased cholesterol biosynthesis has been observed in association with hepatic iron overload, but this was independent of NAFLD (140). Studies in a hyperlipidemic and iron loaded rat model also demonstrated that excess iron significantly increased serum triglycerides and glucose levels but did not affect serum cholesterol concentrations (141). A recent in vitro study has evidenced the inhibition of secretion of apolipoprotein B via a post translational mechanism
by ferritin heavy or light chain leading to the degradation of apolipoprotein in the endoplasmic reticulum (142). These reports provide possible mechanisms connecting iron and lipid metabolism which could contribute to the development of NAFLD.

Iron is also implicated in interfering with insulin signalling leading to impaired glucose metabolism (143). Hyperinsulinaemia and insulin resistance are associated with NAFLD/NASH and are concomitant with increased hepatic iron levels (144). In an insulin resistant (IR) state, glucose uptake by peripheral tissues is reduced and hepatic glucose output remains uninhibited and is a common finding associated with steatosis (97). In keeping with this, increased hepatic iron in patients with NAFLD has also been associated with IR (127, 145, 146). The mechanism by which iron overload may interfere with insulin resistance is yet unknown but there are various proposed mechanisms. Iron induced catalysis of oxidative stress can cause inflammation which has been implicated as a major factor in insulin resistance. TNFα is a common inflammatory mediator in iron induced oxidative stress and NAFLD, and downregulates insulin signalling by reducing expression of glucose transporter 4 (GLUT4) and insulin receptor substrate 1 (IRS1) (147) hence altering insulin uptake and regulation of insulin signalling (148, 149). Serum iron and ferritin may also contribute to insulin resistance in adipocytes by reducing glucose uptake (150) or lipolysis (151). Further evidence for the role of iron in the development of insulin resistance comes from studies of iron depletion by phlebotomy which has led to improved glucose tolerance and improvement of serum alanine aminotransferase levels in patients with NAFLD (152, 153).

Iron also appears to induce inflammation via activation of macrophages and hepatic stellate cells. Iron has been shown to activate phosphatidylinositol-3-kinase (PI3K) mediated activation of inflammatory signalling in macrophages in vitro (154). In another in vitro study, iron accumulation was shown to reduce insulin sensitivity which was reversed by administration of an antioxidant (155). Hepatic stellate cell activation has also been induced by hepatic iron loading in haemochromatosis and this phenotype was reversed after iron removal by phlebotomy (156).

### 1.6.3 Hepcidin regulation and NAFLD

Hepcidin is a crucial regulator of systemic iron homeostasis and it can be speculated that lipid accumulation may alter systemic iron accumulation via regulation of hepcidin expression.

Conflicting data for hepatic and serum hepcidin levels exists in the literature. Serum hepcidin was found to be significantly higher in patients with biopsy proven NAFLD and was an
independent predictor of lipid parameters (157). Another study by Barisani et al also observed dysregulated hepcidin in patients with DIOS and observed a significant correlation of hepcidin expression with total cholesterol, low density lipoprotein (LDL), and triglycerides (158). Hepcidin expression was also increased in NAFLD patients with iron deposition and this study also demonstrated a correlation of hepcidin expression with the extent of iron overload (159).

On the other hand, another study observed a reduction in hepatic hepcidin expression in NASH cohorts. This study demonstrated a positive correlation between hepatic hepcidin expression and levels of serum triglycerides and cholesterol in patients (160). Furthermore, evidence from murine models of diet-induced obesity has also demonstrated a reduction in hepatic hepcidin expression with the onset of steatohepatitis (10, 161). A recent study has also showed that hepcidin is stimulated by insulin in a rodent model of type 2 diabetes (162). While there is contradictory data for hepcidin, expression appears to be correlated with lipid parameters despite the primary role for hepcidin in regulating iron homeostasis.

An alternative theory for the development of iron loading in NAFLD is due to hepcidin independent mechanisms. In keeping with this, a recent study in NASH patients has observed increased duodenal iron absorption despite elevated serum hepcidin. This iron uptake was increased via the upregulation of duodenal divalent metal transporter 1 (DMT1) expression (163).

The reason for iron loading in NAFLD is largely unknown and the evidence provided suggests a role for hepcidin. This represents an avenue to focus further research to understand the underlying mechanisms of iron loading in NAFLD.

To summarise, iron can potentially exacerbate NAFLD pathogenesis through a variety of mechanisms such as the production or ROS, development of insulin resistance, disrupted lipid metabolism and activation of inflammatory signalling pathways (Fig 1.9). Studies have described a role for HFE gene mutations in development of iron overload and the remainder of this review will discuss this co-incidence and its possible role in the development of NASH.
Fig 1.9: Overview of potential mechanisms for hepatic iron deposition and pathways of iron induced NASH pathogenesis. Genetic mutations, diet and altered erythropoiesis can induce iron loading usually by altering the hepcidin-ferroportin axis. Iron loading can consequently cause impairment of insulin signalling, ROS accumulation and increased liver injury. Helicobacter pylori (H. pylori), ferroportin-1 (FP-1), alpha-1 antitrypsin (AAT), red blood cell (RBC), transferrin receptor (TfR), tumour necrosis factor-α (TNFα), reactive oxygen species (ROS). This figure has been reproduced with permission from Hepatol Res, Volume 39, 2009 (164).

1.7 HFE haemochromatosis and NASH

1.7.1 Prevalence
The two most common HFE gene mutations are the p.C282Y and p.H63D which have led to hepatic iron overload. Many studies, predominantly in Caucasian populations have found that HFE mutations commonly co-exist with NAFLD/NASH pathology (7, 8). This association was however infrequent in non-Caucasian populations in Japan (130, 165), Brazil (166), Taiwan (167) and India (168, 169), which can be accounted for by the low incidence rate of these mutations in these regions. A recent meta-analysis examining this association across ethnicities did not find an overrepresentation of the HFE genotype in NAFLD in Caucasian populations.
In spite of these controversies regarding the association of HFE mutations with severity of injury in NAFLD patients, many studies have reported increased iron stores in patients with HFE mutations and NASH (6-9, 125). Homozygous and heterozygous C282Y mutations have been found in patients with NASH and have been associated with an increase in hepatic iron deposition which was positively correlated with the degree of fibrosis (7). Bonkovsky et al. reported that the severity of disease correlated with the HFE mutation in NAFLD but this was independent of HIC (6). Another study showed that hepatic steatosis was found in 41.1 % of haemochromatosis patients and was associated with elevated ALT and serum ferritin, and the progression of fibrosis (9).

1.7.2 Evidence for Hfe and NAFLD co-toxic liver injury from animal models

1.7.2.1 Hepatic lipid handling

An Hfe<sup>-/-</sup> mouse model has been characterised which represents an animal model of hereditary haemochromatosis (171). A study in a Hfe<sup>-/-</sup> mouse model, investigating the role of Hfe inactivation independent of hepatic iron overload has found differential expression of glutathione-S-transferase P1 (GSTP1), liver carboxylesterase 1, selenium binding protein 2 (SBP2) and major urinary proteins 1, 2 and 6 (MUPs) (172). Among these differentially expressed genes, glutathione-S-transferase P1 is involved in TNFα signalling (173). TNFα has a role in iron metabolism and it was hypothesised that induction of GSTP1 in Hfe<sup>-/-</sup> mice might influence iron metabolism by abrogated TNFα signalling. It has been reported that TNFα has a role in abrogating duodenal iron uptake by interfering with FPN, independent of hepcidin, and also by sequestering iron in the spleen (174).

Liver carboxylesterase 1 is involved in cholesterol and fatty acid metabolism (175) and although there is no known function for SBP2, it has been recently implicated in liver fibrogenesis (176), indicative of a role for Hfe<sup>-/-</sup> in disease progression via altered cholesterol and fatty acid metabolism. It is also interesting that three of the proteins (GSTP1, SBP2 and MUP2) that were upregulated in Hfe<sup>-/-</sup> mice have been shown to be downregulated by peroxisome proliferator activated receptor alpha (PPARα), a transcriptional factor which regulates the expression of genes involved in fatty acid β-oxidation and energy homeostasis and has a role in reduction of hepatic steatosis (177). This upregulation suggests a possible role for a process adverse to PPARα activation in Hfe-associated disease progression (172, 178).
Another gene expression profiling study of $Hfe^{-/-}$ mice liver found the downregulation of genes involved in β-oxidation and cholesterol metabolism (179) suggesting a role for $Hfe^{-/-}$ in altered lipid metabolism.

In keeping with these findings, a study from our laboratory in $Hfe^{-/-}$ mice fed a HCD has evidenced the development of NASH associated with an unusual upregulation of lipogenic genes and downregulation of genes involved in fatty acid β-oxidation in spite of accumulating steatosis (10).

1.7.2.2 Oxidative stress

Oxidative stress can be generated by lipid accumulation and by iron overload (102, 180). It is likely that the co-incidence of the two conditions which generate oxidative stress will further exacerbate oxidative injury. Evidence from $Hfe^{-/-}$ mice fed a HCD showed increased expression of hypoxia inducible factor 1α (HIF-1α) and reduced manganese superoxide dismutase (MnSOD) activity which is indicative of increased oxidative stress (10). The results from this study are consistent with a possible role for mitochondrial dysfunction which has been observed in patients with NAFLD (181). This might suggest a role for dysfunctional mitochondria in production of oxidative stress in exacerbating liver injury. There was also an upregulation of genes from the cytochrome P450 family which are upregulated in response to oxidative stress (182) in another study in $Hfe^{-/-}$ mice. Additional evidence for a role of oxidative stress comes from a study of iron and fat loading in vitro where AML12 hepatocytes developed insulin resistance associated with the development of oxidative stress, and the blunted response to insulin stimulus was reversed with the administration of an antioxidant, curcumin (155). A similar observation was evidenced in $Hfe^{-/-}$ mice which developed steatohepatitis when fed a high calorie diet and steatotic injury was reduced when mice were supplemented with dietary curcumin. The reduction in steatosis in this model was also accompanied by a reduction in inflammatory gene serum amyloid A1 ($Saa1$) (unpublished data from our laboratory).

1.7.2.3 Fibrosis

Evidence from an $Hfe^{-/-}$ mouse model has found upregulation of SBP2 which has been implicated in hepatic fibrosis (172). There has also been evidence from an expression profiling study for upregulation of inflammatory response genes: serum amyloid 1, 2 and 3 ($Saa1$, $Saa2$ and $Saa3$) and orosomucoids 1 and 2 ($Orm1$ and $Orm2$) (182). This upregulation of the serum amyloid genes occurred in $Hfe^{-/-}$ mice independent of dietary iron overload. This inflammatory
response in Hfe\textsuperscript{+} mice may potentially be exacerbated with an added insult of accumulation triglycerides in a steatotic liver. Results from a study on Hfe\textsuperscript{+} mice fed a HCD also showed development of severe micro and macrovesicular steatosis, hepatocyte ballooning and inflammatory cell infiltration (10). These mice had greater liver injury evidenced by higher ALT levels – 7-fold higher than the wild type (WT) controls and 6.8-fold higher than the Hfe knockout mice on a normal diet – which was accompanied with perivenular and perisinusoidal fibrosis which was lacking in the control groups. The fibrosis in these mice was confirmed by the upregulation of a panel of pro-fibrogenic markers including collagen 1a1 (Col1a1), collagen 3a1 (Col3a1), collagen 4a1 (Col4a1), alpha-smooth muscle actin (\(\alpha\)-SMA), MMP’s (matrix metalloproteinases) and tissue inhibitor of metalloproteinases (TIMP’s) (10).

1.8 NASH and alcoholic steatohepatitis (ASH)

While the main focus of this thesis is Hfe-associated NAFLD, the discussion of ASH is imperative as it has a common pathophysiology to NASH and it is likely that commonalities in disease pathogenesis exist. Some of these similarities have been discussed below.

ASH is characterized by steatosis as a result of excessive long-term alcohol intake, which results in disturbances of lipid metabolism such as inhibition of fatty acid oxidation and enhanced lipogenesis (183). Although the primary insult for the development of ASH and NASH is different, common mechanistic features in the development of liver injury exist which present clinically as similar serum and histological parameters. Some of these mechanisms are activation of inflammatory processes, production of ROS and consequent oxidative stress, and disruption of lipid metabolism. This suggests that the two disorders have a similar disease pathogenesis (183).

The long term consumption of alcohol can result in liver abnormalities such as simple steatosis or more aggressive injury like scar tissue formation (fibrosis), destruction of liver architecture (cirrhosis) and even hepatocellular carcinoma. Most patients with ASH have significant steatosis in more than 30 % of hepatocytes, and perivenular fibrosis and evidence from animal studies shows that 75 % of hepatocytes from alcohol fed animal’s exhibit steatosis (183).

One process of ethanol degradation, is its conversion to acetaldehyde by cytochrome P450 2E1 (CYP2E1), and rodent models of ALD are characterised by the significant elevation of Cyp2e1 (184). This process of alcohol metabolism by CYP2E1 contributes to the build-up of ROS and
hence promotes the development of steatosis (185). Hepatic CYP2E1 levels are also elevated in patients with NASH and CYP2E1 protein content and activity was positively correlated with the development of liver injury (186-188).

Another commonality in these two disorders is the activation of inflammatory cytokines, primarily TNFα (189). TNFα activation via macrophages in response to gut-derived endotoxins plays a crucial role in the progression of ALD (190). In NASH as well, TNFα has been shown to be overexpressed in both adipose tissue and the liver, suggesting an important role for TNFα in the progression of NASH (183). In keeping with this, it is likely that endotoxins might also play a role in the pathogenesis of NASH (191, 192).

NASH and ASH are major causes of liver cirrhosis and end stage liver disease in the developed world. Both these conditions share similar clinical and pathological characteristics, including steatosis, apoptosis, necroinflammation, progressive fibrosis which may also result in carcinogenesis. Given these similarities, several common pathways for the establishment of liver injury in NASH and ASH have been implicated.

1.8.1.1 Iron overload in ALD

Alcohol consumption is often associated with elevated serum iron. The hepatic iron loading observed is generally modest, with infrequent cases of severe iron loading (193, 194). The iron accumulation in ALD appears to be independent of HFE and other genetic mutations (195).

Alcohol has been shown to downregulate hepcidin expression both in vitro and in vivo and is one possible explanation for iron loading in ALD (196, 197). This downregulation has been reversed by the administration of alcohol metabolising enzymes. Furthermore, iron driven induction of hepcidin has been shown to be blunted by alcohol in models of dietary iron loading (198). There is evidence that alcohol inhibits the binding of STAT3, a positive regulator of hepcidin, to the hepcidin promoter and is proposed to be one mechanism by which alcohol suppresses hepcidin induction (199).

1.9 Summary

In summary, the liver plays a central role in maintenance of the metabolic status of the body. NAFLD represents a condition of impairment of glucose and lipid metabolism and with the additional insult of iron loading the pathogenesis involves a wide spectrum of abnormalities
from triglyceride accumulation and insulin resistance to mitochondrial dysfunction and inflammation. In recent years, studies have concentrated on understanding the pathogenesis of NASH, however the mechanisms underlying injury are largely unknown and effective therapeutic strategies are lacking.

With the increasing incidence of NASH, it almost certainly will become the leading cause of liver transplantation within the decade (Fig 1.10) (4). It has hence become increasingly important to investigate the myriad of events underlying disease pathogenesis and the possible co-toxicities associated with the progression to severe liver injury and a worse clinical prognosis. The primary goal in current hepatology research lies in investigating the ‘trigger’ in the development of steatohepatitis and identification of therapeutic interventions to ameliorate the disease phenotype.

While lifestyle and dietary changes are primary interventions for the reduction in fatty liver disease and obesity these measures are often unsuccessful due to poor adherence (200, 201) and there is an urgent need for effective treatment to avoid the development of advanced liver injury. This need for better therapeutics underlies the significance of understanding the mechanisms of disease development. The understanding of injury mechanisms will not only allow development of novel therapies but will also allow identification of individuals at risk of developing advanced injury and allow early intervention to avoid progression of liver injury.

The above review has described some of the aspects of NAFLD pathogenesis and the co-toxicities associated with HFE- mediated iron loading and has highlighted the complexity and variability of disease pathology. Additionally, this review described the pathogenesis of alcoholic steatohepatitis to highlight the commonalities and differences in induction of injury and the course of establishment of injury.
Fig 1.10: Graphical representation of the projected relative frequencies of NAFLD and HCV as indications for Liver transplantation (LTx). HCV has been the leading cause for liver transplantation. This is likely to change within the decade where NAFLD related complications are predicted to become the leading cause for liver transplantations. This figure has been reproduced with permission from Clin Liver Dis, Volume 13, 2009 (4).

1.10 Hypotheses and aims

The main aim of this thesis was to utilise a ‘global’ approach to identify novel genes in the development of Hfe-haemochromatosis associated steatohepatitis by utilising a transcriptomics approach to identify novel genes involved in the progression of liver injury.

Transcriptomics was performed on messenger RNA from the liver of Hfe<sup>-/-</sup> mice which developed steatohepatitis when fed a high calorie diet.

General hypotheses of this thesis were:

1) Liver injury linked to high calorie diet induced steatosis and Hfe<sup>-/-</sup> related iron overload is associated with the modulation of hepatic gene expression profiles.
2) Fat loading reduces hepcidin expression via the loss of integrity of the iron sensing and inflammatory signalling pathways.

The specific aims were:

1) To identify genes that are differentially expressed between Hfe<sup>-/-</sup> mice fed either chow or a HCD.
2) To investigate expression of candidate genes in different models of chronic liver injury.
3) To develop an *in vitro* model of fat and iron loading and to examine gene expression changes associated with the co-toxic injury.
4) To investigate hepcidin expression and its signalling pathways in fat and iron induced injury.
5) To modulate expression of candidate genes identified from transcriptomics analysis to ascertain their role in disease development.

Addressing the above aims in this thesis will provide more clarity of the underlying pathophysiology in the development of NASH and provide new targets for the development of therapeutics.
Chapter 2  *Materials and Methods*
2.1 Introduction
The methods described in this chapter are for experiments used extensively through the thesis. Individual chapters also describe materials and methods which were used specifically in those chapters.

2.2 Animal maintenance
All wild type C57BL/6J and Hfe<sup>−/−</sup> (supplied initially by William Sly, St. Louis University, MO, USA) mice received humane care under the guidelines and approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee, detailed in the Australian Code of Practice. They were maintained in a temperature controlled environment with 12-hour light-dark cycle with <i>ad-libitum</i> access to food and water. At 8 weeks of age the male mice were fed either chow (n = 10) or a high calorie diet (HCD: SF03-020, see Appendix 2) (n = 10) for 8 weeks or 20 weeks and culled at 16 or 28 weeks of age respectively. These mice were bred and maintained as part of other studies performed by Dr Terrence Tan and Dr Mandy Heritage.

2.3 Liver resection for expression analysis
Mice were anaesthetised with 1 % ketamine/xylazine administered by an intraperitoneal injection and culled at 16 or 28 weeks of age after respective dietary treatments. Following cardiac puncture and exsanguination, the liver was resected, weighed and snap frozen in liquid nitrogen and stored at -80 °C. All initial grading of histology and serum analyses were performed as part of other projects in the laboratory.

2.4 RNA Extractions
Total RNA was extracted using TRIzol Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. Liver tissue/cells were homogenized in TRIzol reagent (1 ml for liver tissue and 500 μl for cells) using a TissueRuptor homogeniser (Qiagen, Hilden, North Rhine-Westphalia, Germany) as per manufacturer’s instructions. The homogenate was incubated at room temperature for 3 min to allow complete dissociation of nucleoprotein complexes. Chloroform (200 μL; Ajax Finechem, Thermo Fisher Scientific, Waltham, MA, USA) was added to the homogenate and shaken vigorously for 15 s followed by incubation at room temperature for 2-3 min. The samples were centrifuged using a Heraeus Pico 17 centrifuge (Thermo Fisher, Langenselbold, Germany) at 12,000 g for 15 min at 4 °C. The top aqueous phase (clear) was transferred to a new tube and 500 μL of isopropanol was added to precipitate the RNA. The samples were gently mixed by inverting the tube 2-3 times
followed by incubation for 10 min at room temperature (RT), and centrifugation at 12,000 g for 10 min at 4 °C. The supernatant was discarded and the RNA pellet was washed in 1 mL of 75% ethanol (prepared in RNase free water), vortexed and centrifuged at 7,500 g for 5 min at 4 °C. The supernatant was discarded and the pellet was air dried at room temperature for 5-10 min. The pellet was resuspended in RNase-free water (Gibco, Life Technologies) and incubated overnight at 4 °C to allow the RNA to dissolve completely. To ensure complete dissolution of RNA, samples were then heated for 10 min at 55-60 °C.

2.5 Quantification of RNA

Purified RNA was diluted 1:100 in MilliQ water and quantified by measuring the absorbance at 260 nm using the Tecan infinite 200 plate reader (Tecan, Männedorf, Switzerland). Purity was assessed using the 260/280 nm absorbance ratio. RNA with a 260/280 ratio ≥ 1.8 was used for downstream experiments. RNA was stored at -80 °C until required.

RNA concentration was calculated using the following formula:

\[
\text{RNA (ng/μL)} = A \times \varepsilon \times df \times P
\]

A = Absorbance at 260 nm, \(\varepsilon\) = Extinction co-efficient (40 M\(^{-1}\)·cm\(^{-1}\)), df = dilution factor (100) and P = 1.733 (derived from the path length of the light and corrects for using a 96-well plate rather than a cuvette).

2.6 cDNA preparation

RNA was treated with DNase I (Invitrogen) to remove any carry-over genomic DNA before reverse transcription. 1 μg of RNA was diluted in 8 μl RNase free water, and 1 μl 10X buffer with MgCl\(_2\) (Invitrogen) and 1 μl DNase I enzyme were added to the RNA and incubated at RT for 15 min. The reaction was stopped by adding 1 μl 25 mM EDTA (Invitrogen) and incubating at 65 °C for 10 min. The DNA-free RNA was mixed with 1 μl 10 mM dNTPs (Invitrogen) and 1 μl oligo dTs (Invitrogen) and heated for 5 min at 65 °C, followed by incubation on ice for 5 min. To this reaction mix, 4 μl 5X first strand buffer (Invitrogen), 1 μl 0.1 M DTT (Invitrogen), 1 μl RNaseOUT (Invitrogen) and 1 μl Superscript III Reverse Transcriptase was added and incubated at 50 °C for 1 hour, followed by an incubation at 70 °C for 15 minutes. The cDNA product was diluted 1 in 20 (or 1 in 5 for cDNA from cells) with RNase-free water and stored at -20 °C until used for real time-quantitative PCR.
To prepare a cDNA standard for use in RT-qPCR experiments, RNA from all the experimental groups, which covers the dynamic range of gene expression, were processed. These samples were combined, not diluted and stored until required.

2.7 Real Time – Quantitative Polymerase Chain Reaction (RT-qPCR)

Real time-quantitative PCR was performed using the Sybr Green method (Qiagen QuantiFAST Sybr). A master mix of 5 μl QuantiFAST Sybr, 0.2 μl forward primer (0.4 μM final concentration), 0.2 μl reverse primer (0.4 μM final concentration) and 2.6 μl RNase free water was prepared and 8 μl was aliquoted per well in a 384-well plate as per experimental layout. A seven step serial dilution (1 in 3) of the undiluted cDNA standard was also prepared. The relative concentration of the standards was expressed in arbitrary values and a linear standard curve was derived to determine the quantity of the target nucleic acid sequence in the sample. To the wells on the 384-well plate, 2 μl of cDNA sample or standard was added in duplicate. Thermal cycling was performed on a ViiA 7 Real-Time PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA, USA) with a hot start at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. A melt curve analysis (95 °C for 15 s, 60 °C for 1 min followed by 95 °C for 15 s) was also performed for quality control.

The quantity of expression of reference genes (Gapdh, B2MG and BTF3) and target genes in the samples was interpolated from the standard curve for the respective genes. The geometric mean of the quantity of expression for the three reference genes was then calculated and this value was used to normalise the quantity of expression of the target genes to provide a relative gene expression for each sample. Primer sequences are outlined in Table 2.1.
Table 2.1: RT-qPCR primers used for expression analysis in mouse tissue.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Forward primer (5’ – 3’)</th>
<th>Reverse primer (5’ – 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acc1</td>
<td>TGAAGACCTTTAAGCCAAATGC</td>
<td>GTTGTTGTTGGGTCTTCCA</td>
</tr>
<tr>
<td>Arsg</td>
<td>CAGTTGGAGTTCTAGGGAGCAGGC</td>
<td>GGTGTGGCAGAATGTGGAGTTAC</td>
</tr>
<tr>
<td>Atoh8</td>
<td>CAACGAGAACAAGCCTCCAGCA</td>
<td>CTTCTGCCATTAGGACTGAC</td>
</tr>
<tr>
<td>B2mg</td>
<td>CTGATACATACGCCTGAGAGTAA</td>
<td>ATGAATCTTCAAGAGCATGTA</td>
</tr>
<tr>
<td>Bax</td>
<td>TTGCTCATAGGTCTTATCCA</td>
<td>GAGACACTGCTAGGCTTCC</td>
</tr>
<tr>
<td>Btf3</td>
<td>TGGCAGCAACACCTCCACC</td>
<td>AGCTAGCCAGTCTTATCAA</td>
</tr>
<tr>
<td>CCL5</td>
<td>CCTGCTGCTTTGCTTACCTCTC</td>
<td>ACACTAGGCGTTGCTCTCAGA</td>
</tr>
<tr>
<td>CoxV</td>
<td>TTCATTGGATTCAGGGCCTT</td>
<td>CTTGATGCGGTGGCTCCCTTCT</td>
</tr>
<tr>
<td>Cpt1a</td>
<td>AGACCAGTGGGAACTCGAACCCTA</td>
<td>TGAAGGTGCTGCTCCACT</td>
</tr>
<tr>
<td>Fas</td>
<td>TACCAAGCCACGCATTCG</td>
<td>TGCTGGTGCACATGAGA</td>
</tr>
<tr>
<td>FPN</td>
<td>GCACGTGCGATCGCAATCCTCC</td>
<td>CTGTTGCTTCTCTTAAGTGAC</td>
</tr>
<tr>
<td>Gpld1</td>
<td>GGAAGCAGAGAGGAATTGAGGC</td>
<td>TCCAAACCAGAGACTGTCCTCC</td>
</tr>
<tr>
<td>Hamp1</td>
<td>TTGCGATACCAAGGCAAGAG</td>
<td>GGATGTTGCTTACAGGTATGTT</td>
</tr>
<tr>
<td>Id1</td>
<td>ACCCTGGAGGCAGGATCA</td>
<td>ACCCTGGACGCAGGATCA</td>
</tr>
<tr>
<td>Ifi2712b</td>
<td>ATGGAAGTGGGAAATTGCAGCAGG</td>
<td>CCAACAGCCACAGGATGATG</td>
</tr>
<tr>
<td>L-Ferritin</td>
<td>GCAACCATCTGACCAACCTC</td>
<td>AGAGATCTGCGCCAGAGACC</td>
</tr>
<tr>
<td>Nrf1</td>
<td>TCCGAAACAGACGAGCACAAGA</td>
<td>TTGGAGGTGAGTAGCAGTT</td>
</tr>
<tr>
<td>Pfk1</td>
<td>CTGGACACCTCCATGGAGGAT</td>
<td>TTGGACACCTCCAGGCTCAGG</td>
</tr>
<tr>
<td>Ppa-α</td>
<td>CATCTGAAGCTTGAAGGCTTT</td>
<td>TCTGACGCTCCAGATCAGCT</td>
</tr>
<tr>
<td>Ppa-γ</td>
<td>CTGCTGAATGCTGTCCTAGTA</td>
<td>TGAGATGGACGCTTCATCTG</td>
</tr>
<tr>
<td>Smad7</td>
<td>GCACAGGACCCCATCTCTC</td>
<td>TCGAGGCTGAGGTCTTCCTC</td>
</tr>
<tr>
<td>Sod2</td>
<td>TACCGGCCAGTACGTGCAGCTG</td>
<td>AGGCTGAAGGAGCAGCTGAGTT</td>
</tr>
<tr>
<td>Sreb1</td>
<td>CACAGGAGGACGACGAGCCTGA</td>
<td>TCAGCCATCCCATAGACAC</td>
</tr>
<tr>
<td>Tfr1</td>
<td>GAGCCAGACCTCCACTCTCT</td>
<td>TGACTGAGATGCGGAAAC</td>
</tr>
<tr>
<td>Tgf-3</td>
<td>ACAACCTTGGACCCCA</td>
<td>GCCACCCGATCCACAGAAGT</td>
</tr>
</tbody>
</table>

Most primer sequences were designed de novo utilising the Primer3 software (V0.4.0: http://bioinfo.ut.ee/primer3-0.4.0/). The generated primer sequences were aligned to the Mus musculus genome using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ascertain specificity to the appropriate gene. Pre-validated primer sequences for Arsg, Gpld1 and Ifi2712b were from Origene (Rockville, MD, USA).

2.8 Protein Extractions

Liver tissue (50-100 mg) was homogenized in 1 ml of freshly prepared cold HES+ extraction buffer (20 mM HEPES, 1 mM EDTA, 250 mM sucrose, 2 mM Na3VO4, 10 mM NaF, 1 mM Na2P2O7, 0.5 mM PMSF, pH7.6) containing Protease Inhibitor Cocktail (1 tab per 50 ml of buffer: Roche, Basel, Switzerland) using a TissueRuptor homogeniser (Qiagen). A hundred microlitres of Triton X-100 (Ajax Finechem, Thermo Fisher Scientific, Waltham, MA, USA) was added to the homogenate.
Cultured cells were pipetted 5-6 times in 100 μL cold HES+ extraction buffer (same as above) containing 1 % Triton X-100 (Ajax Finechem). Tissue homogenates and cell lysates were centrifuged at 12,000 g at 4 ºC for 20 min. The supernatant was collected and stored at -20 ºC.

2.9 Protein Quantification

Protein concentration was determined using the BCA Protein Assay Reagent Kit (Pierce, Thermo Scientific, Rockford, IL, USA). The BCA Working Reagent was freshly prepared by mixing 50 parts Reagent A with 1 part Reagent B. Protein standards were prepared by diluting the provided bovine serum albumin (BSA) solution with MilliQ water according to the manufacturer’s instructions. Samples were diluted 1 in 10 with MilliQ water and 10 μL of each sample or standard was loaded in duplicate to a 96-well plate as per experimental layout. Two hundred microlitres of the BCA Working Reagent was added to all the wells and mixed on a plate shaker. The plate was incubated at 37 ºC for 30 min and absorbance of samples was measured at 540 nm using a Tecan Infinite F200 Plate Reader.

2.10 Western blot

Thirty micrograms of protein was made up in 8 μl MilliQ water and mixed with the loading buffer (2 μl of 5X loading buffer, see appendix for buffer composition) and heated for 5 min at 100 ºC. The samples were loaded into the wells on a 10 % sodium dodecyl sulphate-polyacrylamide gel with 4% stacking gel (SDS-PAGE, see appendix 1) and electrophoresed at 75 V for 10 minutes followed by 150 V until the loading dye reached the bottom of the gel. Polyvinylidene fluoride membrane (PVDF;Biorad, Hercules CA, USA) was prepared by washing in methanol for 30 s followed by 5 min in cold transfer buffer (Table 2.3). After electrophoresis, the gel was carefully separated from the plates and placed onto the PVDF membrane and sandwiched between filter paper and sponge, making sure to remove all air bubbles. The sandwich was transferred to a transfer cassette of a wet blot apparatus (The Mini Trans-Blot, Biorad), making sure the PVDF was on the clear side of the cassette. Proteins were transferred at 100 V for 60 min in the transfer chamber with cold transfer buffer and an ice block to maintain the temperature.

After completion of the transfer, the PVDF membrane was washed in 1X TBS-Tween 20 0.1 % (TBS-T) for 5 min. To prevent non-specific binding the membrane was incubated with blocking buffer (10 % skim milk powder in 1X TBS-T 0.1%) for 1 hr at RT. The primary antibody for the respective proteins was diluted to the optimised concentration (as per Table 2.2) using the blocking buffer. The membrane was incubated with primary antibody overnight.
at 4 °C. The membrane was washed 3 times for 5 minutes with 1X TBS-T and incubated with the respective horse-radish peroxidase labelled secondary antibody diluted in blocking buffer (Table 2.2) for 90 min at RT. The membrane was washed 3 times for 5 minutes with 1X TBS-T and once with TBS (no Tween 20). The West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, Massachusetts, USA) was used to visualise the bands. The reagents were mixed 1:1 and exposed to the membrane: chemiluminescence was measured using the 4000MM pro Image Station (Carestream Health, Inc., Rochester, NY, USA). The protein quantities were determined by densitometry using the Carestream molecular imaging software (v5.3.2, Carestream Health) and expressed relative to GAPDH.

Table 2.2: List of antibodies used for western blotting.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Size (kDa)</th>
<th>Supplier</th>
<th>Primary Ab concentration</th>
<th>Secondary Ab concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arsg</td>
<td>57</td>
<td>Australian Biosearch</td>
<td>1:500</td>
<td>Goat anti-rabbit HRP 1:100,000</td>
</tr>
<tr>
<td>Gapdh</td>
<td>34</td>
<td>MAB374, Millipore</td>
<td>1:150,000</td>
<td>Goat anti-mouse HRP 1:200,000</td>
</tr>
<tr>
<td>Gpld1</td>
<td>93</td>
<td>Santa cruz</td>
<td>1:200</td>
<td>Goat anti-mouse HRP 1:20,000</td>
</tr>
<tr>
<td>HA</td>
<td>Size of tagged protein</td>
<td>Santa cruz</td>
<td>1:1000</td>
<td>Goat anti-mouse HRP 1:50,000</td>
</tr>
<tr>
<td>Ifi2712b</td>
<td>30</td>
<td>Gift from Dr Liao</td>
<td>1:20,000</td>
<td>Goat anti-rabbit HRP 1:50,000</td>
</tr>
<tr>
<td>L-Ferritin</td>
<td>20</td>
<td>MP Biomedical</td>
<td>1:10,000</td>
<td>Goat anti-mouse HRP 1:200,000</td>
</tr>
<tr>
<td>pAkt</td>
<td>56, 62</td>
<td>sc-7985-R, Santa Cruz</td>
<td>1:1000</td>
<td>Goat anti-rabbit HRP 1:100,000</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>79, 86</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Goat anti-rabbit HRP 1:100,000</td>
</tr>
<tr>
<td>SMAD1/5/8</td>
<td>60</td>
<td>Cell Signalling</td>
<td>1:1000</td>
<td>Goat anti-rabbit HRP 1:100,000</td>
</tr>
<tr>
<td>Syndecan-1</td>
<td>75, 25</td>
<td>Abcam</td>
<td>1:1000</td>
<td>Goat anti-mouse HRP 1:20,000</td>
</tr>
<tr>
<td>Tfr1</td>
<td>105</td>
<td>Invitrogen</td>
<td>1:250</td>
<td>Goat anti-mouse HRP 1:200,000</td>
</tr>
</tbody>
</table>

2.11 Statistical analysis

Statistical analysis was performed using the IBM SPSS statistics v22 (IBM Corp, Armonk, NY, USA) or GraphPad Prism v6.0 (La Jolla, California, USA). Differences were considered significant at $p \leq 0.05$. Detailed statistical analyses performed for specific experiments are outlined in respective chapters.
Chapter 3  Transcriptomic Analysis of an Hfe Knockout Model of Steatohepatitis
3.1 Introduction

With the increasing prevalence of steatohepatitis in the population and its co-incidence with HFE mutations (6, 8) it is becoming increasingly important to understand the molecular mechanisms underlying iron and fat co-toxicity. A large number of factors – lipid metabolism, oxidative stress, insulin resistance, mitochondrial dysfunction, inflammation and fibrogenesis – are involved in liver disease progression. In this project, a global approach has been utilised to identify novel factors that may be involved in disease progression.

Transcriptomics is the study of the dynamic population of messenger RNA in a cell/tissue, which helps to identify the metabolic state of the sample. It helps interpret the functional elements of the genome and reveals molecular constituents of the sample to gain a better understanding of disease and development. Given the dynamic nature of the transcriptome, transcriptomics is an essential tool in examining different physiological conditions by quantifying gene expression. Transcriptomics has been widely used as a tool for biomedical research but until recently the technology has been limited to the use of expression microarrays. Microarrays have been a useful technique for decades however their role is restricted to detecting transcripts with a known sequence. Messenger RNA sequencing (mRNA-seq) is a new high throughput sequencing technology which produces millions of sequence reads per sample. This approach enables the quantification of gene expression with a wider dynamic range of detection in comparison with microarrays. It provides coverage of all expressed transcripts, including unknown or novel genes and also allows detection of different isoforms of a gene (202, 203).

A few high throughput sequencing platforms are available all of which utilise different chemistries for sequencing. The Ion Torrent Personal Genome Machine (Thermo Fisher Scientific, Waltham, MA, USA) which utilises a ligase-enhanced genome detection technology has been utilised for sequencing in this project. Briefly, an mRNA population is converted to a library of cDNA with adaptors attached to both ends. These are then amplified and sequenced while maintaining strand specificity to yield sequence reads which are approximately 200 bp long. During the sequencing process, homo-nucleotide preparations flood the chip, when a nucleotide is complementary, it hybridises, a process which results in the release of a single hydrogen atom per nucleotide incorporation. The Ion sequencing chip captures the change in voltage caused by the released hydrogen ion (H^+). If two or more nucleotides are incorporated there is a proportional increase in the voltage allowing accurate estimation of homopolymer
chains (204). After sequencing, the reads are aligned to a reference genome to generate a map of the transcriptome. The mapped reads are annotated against a transcriptome database and counted to obtain the number and density of reads aligned to a particular exon, transcript or gene (depth of sequencing).

mRNA-seq detects differentially expressed genes utilising the ‘count’ data providing a more precise measurement of the transcript levels and a greater dynamic range of detection compared to microarrays. mRNA-seq also enables detection of genes which are expressed at low levels which may be physiological relevant (202, 205) and avoids the drawback of microarray technology in avoiding the high background ‘noise’ associated with cross-hybridisation artefact resulting from hybridisation of probes to non-specific targets.

In spite of its various advantages, mRNA-seq poses challenges with regard to dependence on the depth of sequencing to enable the detection and quantitation of low abundance transcripts. Not all mRNA-seq analyses yield the same level of accuracy, with variability in uniformity across the transcriptome resulting from differences in library preparation method and consistency, influencing sequencing results. Additional challenges are posed by large transcriptomes such as mouse and human, the analysis of which requires costly computational resources to map reads to the genome.

mRNA-seq produces large amounts of data and requires bioinformatics for its processing and analysis of the large data sets can be time consuming, complicated and costly. mRNA-seq is a relatively new technology and the statistics of the ‘count’ data is not fully understood and data analysis methodologies are still evolving. Hence for most experiments, mRNA-seq data is transformed to a continuous dataset to enable use of appropriate statistical tools for further downstream analysis (206). The method of normalisation is also still in evolution, with several methods currently utilised in publication. This process needs to be more streamlined and currently poses a challenge in the analysis of mRNA-seq data. Current methodologies for mRNA-seq data analysis include transformation to a continuous dataset prior to further analysis. This methodology has been utilised in this study and has been described in detail in the results (section 3.5.1) of this chapter.

In this study mRNA-seq has been employed to characterise the liver gene expression profile in \( Hfe^{−/−} \) mice fed a HCD to identify molecular pathways and gene expression changes associated with the development of steatohepatitis. In order to explore differential expression of genes that will influence the development of liver injury we studied \( Hfe^{−/−} \) mice fed HCD since these
animals have the most extensive injury. The $Hfe^{-/-}$ mice fed Chow, with low or no injury and a genetically identical background were used as the control group. The histology and injury parameters for these animals have been outlined in detail in section 3.4.1 of this chapter.

3.2 Hypothesis

Fatty liver injury associated with ingestion of a high calorie diet in $Hfe^{-/-}$ mice is associated with the modulation of hepatic gene expression profiles.

3.3 Aims

The specific aims were:

1) To sequence a cDNA library of liver mRNA utilising the Ion Torrent Personal Genome Machine (PGM).
2) To identify genes that are differentially expressed between $Hfe^{-/-}$ mice fed either chow or a HCD.
3) To validate the differentially expressed genes using RT-qPCR in $Hfe^{-/-}$ and wild type (WT) mice on the respective diets (high calorie diet or chow).
4) To find expression patterns/interactions/clusters within the differentially expressed genes.
3.4 Materials and methods

3.4.1 Animal maintenance and histological characterisation of mice

Animals utilised in this study have been previously characterised as part of other studies in our laboratory by Dr Terrence Tan (10) and Dr Mandy Heritage (unpublished data). Liver tissue from these studies has been utilised for mRNA-seq, validation and functional studies in this project and are explained briefly below.

Hfe<sup>−/−</sup> and wild type (WT) mice were fed respective diets from 8 weeks of age. In one study the animals were fed either chow or a HCD for a further 8 weeks and culled at 16 weeks of age, in a second study mice were fed either chow or HCD for 20 weeks and culled at 28 weeks of age (Fig 3.1). Histological analyses of liver tissue were performed in a double blinded fashion by independent pathologists, Dr Andrew Clouston and Dr Catherine Campbell (Envoi Pathology, Brisbane, QLD, Australia) and liver function was also measured as an indicator of injury (Table 3.1). The mice fed chow, irrespective of genotype or duration of feeding, had normal histology and serum alanine aminotransferase (ALT). The WT mice fed HCD for 8 weeks developed simple steatosis with normal serum ALT, the Hfe<sup>−/−</sup> mice however, developed NASH with early fibrosis and high levels of serum ALT. By 20 weeks, most of the WT mice fed a HCD developed steatohepatitis similar to the Hfe<sup>−/−</sup> mice fed HCD but with lower serum ALT. It was also noteworthy that Hfe<sup>−/−</sup> mice fed HCD for 20 weeks did not display increased severity of injury: steatosis and fibrosis indices remained unchanged, in comparison to Hfe<sup>−/−</sup> mice fed HCD for 8 weeks, suggesting a plateauing of the injury. These mice also displayed reduced hepatic iron concentrations. As previously described in the literature review (Chapter 1, section 1.6.1), there is some evidence for iron loading associated with NASH. Contradictory to this, hepatic iron concentrations were lower in livers with steatohepatitis, independent of the genotype of the mice. Other studies have demonstrated decreased hepatic iron stores in mice and implicated inflammation in this phenomenon (207). Furthermore, a study by Britton et al (Physiological Reports, In press) has shown that while HIC was lower in HCD fed mice, the HIC/hepcidin ratio was appropriate and the low hepatic iron was independent of hepcidin expression.

3.4.2 Liver resection for expression analysis

Livers were resected as described previously in Chapter 2, section 2.3.
Fig 3.1: Schematic representation of the feeding regimen for the animals used in this project. Eight-week old wild type and Hfe\(^{-/-}\) mice were provided either chow or a high calorie diet (HCD) for either 8 weeks or 20 weeks.

Table 3.1: Histology and liver function analysis of WT and Hfe\(^{-/-}\) mice fed either chow or HCD.

<table>
<thead>
<tr>
<th>A</th>
<th>Wild type</th>
<th>Chow (n=9)</th>
<th>High calorie diet (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology Diagnosis</td>
<td>Normal</td>
<td>Steatosis</td>
<td></td>
</tr>
<tr>
<td>(% Stenosis in Liver)</td>
<td>0 (0-0)</td>
<td>10 (5-95)</td>
<td></td>
</tr>
<tr>
<td>Fibrosis Stage</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td></td>
</tr>
<tr>
<td>ALT (u/l)</td>
<td>21</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>HIC (μMol/g tissue)</td>
<td>23.15 ± 1.43</td>
<td>25.08 ± 3.01</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Wild type</th>
<th>Chow (n=9)</th>
<th>High calorie diet (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology Diagnosis</td>
<td>Normal</td>
<td>Steatosis (3)/ Steatohepatitis (6)</td>
<td></td>
</tr>
<tr>
<td>(% Stenosis in Liver)</td>
<td>0 (0-1)</td>
<td>85 (25-100)</td>
<td></td>
</tr>
<tr>
<td>Fibrosis Stage</td>
<td>0 (0-10)</td>
<td>10 (0-10)</td>
<td></td>
</tr>
<tr>
<td>ALT (u/l)</td>
<td>25 (19-37)</td>
<td>98 (25-180)</td>
<td></td>
</tr>
<tr>
<td>HIC (μMol/g tissue)</td>
<td>16.2 ± 0.9</td>
<td>9.7 ± 1.2</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hfe(^{-/-})</th>
<th>Chow (n=6)</th>
<th>High calorie diet (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology Diagnosis</td>
<td>Normal</td>
<td>Steatohepatitis</td>
</tr>
<tr>
<td>(% Stenosis in Liver)</td>
<td>0 (0-0)</td>
<td>95 (95-100)</td>
</tr>
<tr>
<td>Fibrosis Stage</td>
<td>0 (0-0)</td>
<td>1 (0-1)</td>
</tr>
<tr>
<td>ALT (u/l)</td>
<td>26</td>
<td>185</td>
</tr>
<tr>
<td>HIC (μMol/g tissue)</td>
<td>42.06 ± 14.10</td>
<td>27.26 ± 1.54</td>
</tr>
</tbody>
</table>

A) Mice culled at 16 weeks after 8 weeks of feeding B) Mice culled at 28 weeks after 20 weeks of feeding. Alanine transaminase (ALT), hepatic iron concentration (HIC). Fibrosis stage 1 describes perivenular, perisinusoidal and pericellular fibrosis with further classification of ‘a’ for delicate perisinusoidal fibrosis and ‘b’ for dense perisinusoidal fibrosis. The values are represented as median (range) or mean ± SD (10) and unpublished data from our laboratory.

3.4.3 **Messenger RNA library preparation**

The overall procedure of cDNA library preparation from the purified poly(A) RNA has been outlined in Fig 3.2 and has been described in detail below.
3.4.3.1 Total RNA extraction and purification

RNA was extracted from liver tissue using Qiazol reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 15-20 mg of liver tissue was homogenised in 700 μl Qiazol and incubated for 5 min at room temperature to allow dissociation of nucleoprotein complexes. To the homogenate, 140 μl chloroform was added and vigorously shaken for 15 s followed by incubation for 2-3 min. The homogenate was centrifuged for 15 min at 12,000 g at 4 °C. After centrifugation, the upper aqueous phase was carefully separated without disturbing the organic phase. Ethanol (1.5 volumes) was added to the aqueous phase and 700 μl was transferred to a column (Qiagen, miRNeasy kit) for purification of total RNA. The column was centrifuged for 15 s at ≥8000 g at room temperature and was repeated with the remaining homogenate. The column was washed using 700 μl of buffer RWT (Qiagen, miRNeasy kit) and centrifuged for 15 s at ≥8000g at room temperature followed by two washes with 500 μl buffer RPE and centrifuged once as before, followed by another spin for 2 min. The flowthrough was discarded. The column was centrifuged at full speed (16,000 g) for 1 min to eliminate carryover of buffer. RNA was eluted from the column using 30 μl RNase free water and centrifuging for 1 min at ≥8000g at room temperature. This step was repeated with an additional 30 μl RNase-free water to maximise the RNA yield from the column.

3.4.3.2 Total RNA quantification

Purified RNA was quantified by measuring the absorbance at 260 nm using the Tecan spectrophotometer (Tecan, Männedorf, Switzerland) and purity was assessed by determining the 260/280 nm absorbance ratio. The quality of RNA was also analysed on the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples were diluted to fall within the detectable range for the RNA 6000 Pico kit (Agilent Technologies) and loaded onto a chip with a RNA ladder. Following electrophoresis the RNA Integrity Number (RIN) was calculated by an algorithm patented by Agilent technologies. The RNA used for further experimentation had a RIN greater than 7 (on a scale of 1 to 10, where 1 = most degraded and 10 = most intact).

3.4.3.3 Poly (A) RNA purification

Up to 50 μg of total RNA was used for poly (A) RNA purification using the Dynabeads mRNA Direct Micro Kit (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The appropriate volume of ERCC (External RNA Controls Consortium) Spike-in mix (Ambion, Life Technologies) – a control for downstream experiments – was added to the RNA samples and the volume was made up to 300 μl using
nuclease free water. The diluted RNA was heated for 2 min at 70 °C and 300 μl lysis buffer was added to each sample. The appropriate volume of Dynabeads was added to the sample, mixed and left to incubate for 5 min. The sample was placed on a magnetic stand (DynaMag-2 Magnet, Thermo Fisher Scientific) and when the solution appeared clear, the supernatant was discarded and the beads (bound to mRNA) were mixed with 600 μl of wash buffer A. The sample was then kept on the magnetic stand for separation and the procedure was repeated with 300 μl of wash buffer B. To the separated beads, 90 μl of pre-warmed (80 °C) nuclease-free water was added and incubated for 30 s at room temperature. The eluted mRNA was rebound to the dynabeads by adding 90 μl of lysis buffer and incubating for 5 min. The beads were separated by placing on a magnetic stand and the supernatant was discarded and the beads were washed with wash buffer A followed by a wash with wash buffer B. The appropriate volume of pre-warmed (80 °C) nuclease-free water was added to the beads and incubated for 30 s. The beads were separated by placing on a magnetic stand and the eluent containing the mRNA was transferred to a new tube and stored for subsequent experiments.

3.4.3.4 RNA fragmentation

Purified mRNA was used for preparation of the mRNA library for use on the Ion Torrent Personal Genome Machine using the Ion Total RNA-seq Kit v2 (Life Technologies). Up to 500 ng of poly(A) RNA was fragmented by RNase III enzymatic action. The reaction mix containing 8 μl poly(A) RNA, 1 μl 10X RNase III reaction buffer and 1 μl RNase III was incubated in a thermal cycler at 37 °C for 10 min, after which 20 μl of nuclease-free water was added immediately to the reaction mix and placed on ice.

3.4.3.5 Purification of fragmented RNA

The nucleic acid beads from the magnetic bead module of the Ion RNA-seq Kit v2 (Life Technologies) was vortexed and 5 μl transferred to a new tube. The beads were mixed with 90 μl of binding solution concentrate and 30 μl of the fragmented RNA. To this, 150 μl of 100 % ethanol was added and allowed to incubate for 5 min at room temperature. The beads were separated from the solution by placing on a magnetic stand and supernatant discarded. The beads were washed with 150 μl of wash solution concentrate and incubated at room temperature for 30 s. The supernatant was discarded and the beads air-dried for 2 min. The beads were then mixed with 12 μl of pre-warmed (37 °C) water and incubated for 1 min to allow elution of the fragmented RNA. The beads were separated by placing on the magnetic stand and the eluent was collected.
3.4.3.6 **Assess yield and size distribution of fragmented RNA**

The Qubit RNA assay kit (Invitrogen, Life Technologies) was used to quantitate the yield of fragmented poly(A) RNA on the Qubit Fluorometer (Invitrogen, Life Technologies) according to manufacturer’s instructions. Fragmented RNA samples were diluted with nuclease-free water to fall within the detectable range for the Qubit RNA assay kit (Invitrogen, Life Technologies). A working solution was prepared by mixing 1 part working reagent with 199 parts Qubit RNA buffer. Standards were prepared by diluting 1:20 with the prepared working solution and appropriate volume of each sample was made up to 200 μl with working solution. The samples were read following a 2 min incubation at room temperature and the concentration of RNA was calculated by the Qubit Fluorometer (Invitrogen, Life Technologies).

Size distribution of the fragmented RNA was also assessed using the Agilent Bioanalyser RNA 6000 pico kit (Agilent Technologies) according to the manufacturer’s instructions. The peaks were visualised and average size assessed.

3.4.3.7 **cDNA preparation and purification**

Ninety nanograms of fragmented RNA was added to 2 μl of Ion adaptor mix v2, 3 μl of hybridisation solution and volume was made up to 5 μl with nuclease free water. This reaction mix was incubated in the thermal cycler at 65 °C for 10 min followed by 30 °C for 5 min to hybridize the adaptor to the fragmented RNA. To the hybridisation mix 10 μl of 2X ligation buffer and 2 μl of ligation enzyme mix was added and incubated in a thermal cycler with an open lid (to avoid the heated lid of the thermocycler, alternatively one could turn off the heated lid setting or set the temperature of the lid to match that of the block) at 30 °C for 30 min. The adaptor-ligated RNA was mixed with a reverse transcription master mix containing 2 μl nuclease free water, 4 μl 10X reverse transcription buffer, 2 μl 2.5mM dNTP mix and 8 μl ion reverse transcription primer v2 and incubated in a thermal cycler with a heated lid at 70 °C for 10 min followed by a snap cool on ice. Lastly, 4 μl of 10X superscript III enzyme mix was added to the ligated RNA and reverse transcription was performed in a thermal cycler with a heated lid at 42 °C for 30 min.

The Magnetic bead module of the Ion RNA-seq Kit v2 (Life Technologies) was then used to purify the prepared cDNA according to manufacturer’s instructions. The nucleic acid beads were mixed using a vortex, 10 μl transferred to a new tube and mixed with 120 μl of binding solution concentrate. The reverse transcription mix was mixed with 60 μl of nuclease free water and the full volume was added to the nucleic acid beads and binding solution mix. To this, 125
μl of 100 % ethanol was added and allowed to incubate for 5 min at room temperature. The beads were separated from the solution by placing on a magnetic stand for 5-6 min and the supernatant was discarded. The beads were washed with 150 μl of wash solution concentrate and incubated at room temperature for 30 s. The supernatant was discarded and the beads air-dried for 2 min. The beads were then mixed with 12 μl of pre-warmed (37°C) nuclease free water and incubated for 1 min to allow elution of cDNA. The beads were separated by placing on the magnetic stand for 1 min and the eluent was collected.

3.4.3.3 cDNA amplification and purification
Six microlitres of the purified cDNA was mixed with 45 μl Platinum PCR SuperMix high fidelity (Life Technologies), 1 μl Ion 5’ PCR primer and 1μl Ion 3’ PCR primer and amplified in the thermal cycler using the following cycling conditions: 2 min at 94 °C, 2 cycles of 94 °C for 30 s, 50 °C for 30 s and 68 °C for 30 s, 14 cycles of 94 °C at 30 s, 62 °C at 30 s and 68 °C at 30 s followed by a final step of 68 °C for 5 min. The amplified cDNA was then purified utilising the magnetic bead clean-up module of the Ion RNA-seq Kit v2 (Life Technologies). The nucleic acid beads were mixed using a vortex and 10 μl transferred to a new tube and mixed with 180 μl of Binding solution concentrate and 53 μl of amplified cDNA. To this, 130 μl of 100 % ethanol was added and allowed to incubate for 5 min at room temperature. The beads were separated from the solution by placing on a magnetic stand for 5-6 min and supernatant discarded. The beads were washed with 150 μl of wash solution concentrate and incubated at room temperature for 30 s. The supernatant was discarded and the beads air-dried for two min. The beads were then mixed with 15 μl of pre-warmed (37 °C) nuclease free water and incubated for 1 min to allow elution of the fragmented RNA. The beads were separated by placing on the magnetic stand for 1 min and the eluent was collected.

3.4.3.9 Assessment of size distribution of amplified cDNA and calculation of template dilution factor
The Qubit dsDNA HS assay kit (Invitrogen, Life Technologies) was used to quantitate the yield of amplified cDNA on the Qubit Fluorometer (Invitrogen, Life Technologies) according to the manufacturer’s instructions. Briefly, a working solution was prepared by mixing 1 part Qubit dsDNA HS reagent with 199 parts Qubit dsDNA HS buffer. Standards were prepared by diluting 1:20 with the prepared working solution and appropriate volume of each sample was made up to 200 μl with working solution. Sample fluorescence was read following a two min incubation at room temperature and concentration was calculated by the Qubit Fluorometer (Invitrogen, Life Technologies). A smear analysis was performed to quantify the percentage of
cDNA that was ≤ 160 bp (base pairs). The median peak size (bp) and molar concentration of the cDNA were also determined using the Agilent software. The template dilution factor was calculated to yield $210 \times 10^6$ molecules of template per 20 μl reaction. The library concentration was calculated from the smear analysis and the conversion factor of 8.3 nM = 5 $\times$ 10⁹ molecules/μl was used as described in the equation below.

$$\text{Template dilution factor} = \left(\text{Library concentration in nM}\right) \times \left[\frac{5\times10^9\text{molecules per } \mu l}{8.3\text{nM}}\right] \times \left[\frac{20\mu l}{210\times10^6\text{molecules}}\right]$$

For example: For a library concentration of 4 nM, the template dilution factor will be 229. Hence 1 μl of the 4 nM library diluted in 228 μl of nuclease free water (1:229 dilution) will yield approximately $210 \times 10^6$ molecules of template per 20 μl reaction.

### 3.4.3.10 Clonal amplification of library by emulsification PCR

The Ion One Touch Instrument (Life Technologies) was setup and initialised as per the manufacturer’s instructions. Using the Ion one touch 200 kit the amplification solution was prepared by mixing 280 μl nuclease free water, 500 μl Ion one touch 2X reagent mix, 100 μl Ion one touch enzyme mix and 20 μl of diluted library (prepared as per formula above). Ion sphere particles (ISPs) from the kit were mixed by using a vortex and 100 μl added to the amplification solution. The amplification solution was then loaded into a filter assembly, installed on the Ion one touch instrument and run. After completion of the run, the template positive ISPs were washed using the Ion one touch wash solution and retained to enrich the template positive ISPs using the Ion one touch ES.

### 3.4.3.11 Enrichment of template-positive ISPs

Dynabeads MyOne streptavidin C1 beads, used to enrich the template positive ISPs, were washed and 130 μl loaded on an 8 well strip along with 300 μl of the melt-off solution – 865 μl nuclease free water, 125 μl 1M sodium hydroxide (NaOH) and 10 μl of 10% Tween 20 in nuclease free water. The total volume of unenriched sample (100 μl) was also loaded and the run was performed. The enriched ISPs were washed and retained to load onto the 318 chip to be sequenced. A quality check at this stage was performed to assess enrichment efficiency of the template positive ISP’s utilising the Ion sphere quality control kit (Life Technologies).
3.4.3.12 Library sequencing

The template positive ISPs were annealed to sequencing primers in a thermal cycler for 95 °C for 2 min followed by 37 °C for 2 min utilising a heated lid. After annealing the primers, the instrument was cleaned and initialised following manufacturer’s instructions. The 318 chip was washed by applying gently pressure on the pipette and avoid the introduction of air bubbles. Once washed, the chip was centrifuged to remove the excess wash buffer and the sample was loaded onto the chip by dialling down the pipette to a lower volume to allow gentle and slow release of the sample onto the chip and to facilitate a high loading efficiency.

3.4.4 Bioinformatics and statistical analysis

The sequence data was processed by the Ion Torrent Suite v3.2 and aligned to the mouse reference genome mm10 (https://genome.ucsc.edu/cgi-bin/hgGateway?db=mm10). Data was normalised by the reads per kilobase of exon model per million mapped reads (RPKM) method to account for variability both between and within samples. Upper quartile normalisation was also performed to account for overrepresentation of highly expressed genes. The data was converted to a normal distribution by performing a log2 transformation. Differentially expressed genes were identified using a one-way ANOVA (Partek Genomic Suite v6.6, Partek Inc., St Louis, Missouri, USA) at a p-value ≤ 0.05 and fold change (FC) of ± 1.5 assuming the Benjamini and Hochberg’s criterion for multiple testing to account for the false discovery rate (FDR). In order to perform gene ontology enrichment a larger data set with less stringent filtering criteria was utilised (p-value (FDR) ≤ 0.1 and FC ±1.5) (Fig 3.3).

3.4.5 Enrichment analysis

Gene ontology (GO) enrichment (Partek genomic suite v6.6) was utilised to cluster genes based on functional categories. Differentially expressed genes with a p-value (FDR) ≤ 0.1 and FC ± 1.5 were utilised to identify overrepresented GO groups.

3.4.6 Gene expression analysis

The most significantly differentially expressed genes were validated by real time-quantitative polymerase chain reaction (RT-qPCR) using all mice in each group. cDNA was prepared from total RNA as previously described (Chapter 2, Section 2.5). Primers were designed in Primer 3 v0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/, Table 3.2). Thermal cycling was carried out using the ViiA 7 Real-Time PCR system (Applied Biosystems, Life Technologies) with a hot start at 95 °C for 5 min followed by 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. The primers utilised are enlisted in Table 3.2. Target gene
expression was normalised against the geometric mean of expression of the reference genes: 
\textit{Gapdh, B2mg and Btf3}.

\textbf{Fig 3.2: Schematic of the process for cDNA library preparation for sequencing.} Liver RNA was extracted, poly(A) RNA purified and fragmented. The adaptors were then ligated to the RNA, reverse transcribed to cDNA which was clonally amplified and sequenced. Quality analysis/Quality check (QA/QC).
Raw sequence reads
(Ion Torrent Personal genome Machine)

\[ \downarrow \]

Removal of adapter sequences and low quality reads
(Phred score > 10)

\[ \downarrow \]

Alignment to reference genome \( mm10 \)
(Tmap aligner)

\[ \downarrow \]

Aligned reads annotated
(Refseq)

\[ \downarrow \]

Data normalised
(RPKM + Upper quartile)

\[ \downarrow \]

Differential Expression analysis
(1-way ANOVA)

\[ \downarrow \]

Differentially expressed genes
\( p(\text{FDR})<0.05 \text{ FC} = \pm 1.5 \)

**Fig 3.3: Overview of mRNA-seq analysis pipeline to detect differentially expressed genes.**

Score for the probability of correctly calling the base on a sequence read (Phred score), Torrent mapping alignment programme (Tmap), Reads per kilo base of exon per million mapped reads (RPKM), false discovery rate (FDR), fold change (FC).

### 3.4.7 Statistical analysis

Relative expression data from RT-qPCR analysis was log transformed, \( \log_{10}(x) + 1 \), to transform the data into a normal distribution. The log transformed data was subjected to a 2-way analysis of variance (ANOVA) and the significant effects of the diet or genotype at \( p \leq 0.05 \) were considered significant and have been reported. In experiments where an interaction of the respective treatments was found significant, the individual effects are not reported. In this case, Holm-Sidak’s post-hoc test was performed and the differences between individual groups are represented. Where two independent groups were compared, a student t-test was performed. The difference between the groups was found significant at \( p \leq 0.05 \).

All statistical analysis was performed using the IBM SPSS statistics v22 (IBM Corp, Armonk, NY, USA) and graphs were generated using GraphPad prism v6.0 (La Jolla, California, USA).
Table 3.2: Mouse primer sequences utilised for RT-qPCR validation of differentially expressed genes.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Forward primer (5' – 3')</th>
<th>Reverse primer (5' – 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldh1i1</td>
<td>CACCTTCGGGACTTCTATC</td>
<td>CTGGTAGGGGATTTGGAAGAG</td>
</tr>
<tr>
<td>Aldh3a2</td>
<td>GGGCTTCAGAATCAGCAGGA</td>
<td>ACATCCCTGGTGTACACACAC</td>
</tr>
<tr>
<td>Aldh6_ps1</td>
<td>CCCCAAGCTGTCTCTTTCAG</td>
<td>GGTGTCTCTCACATCTAGCA</td>
</tr>
<tr>
<td>Arsg</td>
<td>AGTCCAACGAAGACAGACAC</td>
<td>CTATCCAGGTTGGGAGATG</td>
</tr>
<tr>
<td>Ccnd1</td>
<td>GGATGTCAGGGGAAGAGGAGTG</td>
<td>CAGCGAAACACACGAGAAGAAG</td>
</tr>
<tr>
<td>CD 36</td>
<td>ACAGACGCAGCCTCTCTTTTC</td>
<td>CAGATCCGAACAGACGATG</td>
</tr>
<tr>
<td>Cidec</td>
<td>CATCAGAAACAGCAGGAAGAAG</td>
<td>AGGCAGCAATAAGCTGCTG</td>
</tr>
<tr>
<td>Gm4956</td>
<td>TGGGATGACTTTGGTGCAGA</td>
<td>TACTGTTGTAGGCTGGTTGA</td>
</tr>
<tr>
<td>Gpld1</td>
<td>TGTTGGGAATACCTGCTCTTC</td>
<td>CAAATAGGACCAGGACCACCA</td>
</tr>
<tr>
<td>Hsd17b13</td>
<td>CGGTATCCCAAACCTGCTGTC</td>
<td>GCAACTTCTTCCGGCTCTAA</td>
</tr>
<tr>
<td>Hsd3b5</td>
<td>GCTTCCAGACAGACGATCGTCTAG</td>
<td>TTTTGCTCAGCCACAACAGACTG</td>
</tr>
<tr>
<td>Ifi27i2b</td>
<td>ATGGAGGTGGAATTTGCAGCAGG</td>
<td>CCAACAGCCACAGGATGATGT</td>
</tr>
<tr>
<td>Lipa</td>
<td>GCTCCCCGTGTTGCTCTCTCA</td>
<td>CCAACACAGATGATGATG</td>
</tr>
<tr>
<td>Plin2</td>
<td>CTCAGGAGGAGCTGGAGATG</td>
<td>TATAAGCAGCAGAAGCAGAG</td>
</tr>
<tr>
<td>Sconn1a</td>
<td>TGCTCCTGTGCTTTCAGCAGC</td>
<td>TCAATGGGGCTGTCTCTCG</td>
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<tr>
<td>Slco1a1</td>
<td>CAAGGGGAGTTGGTTCTCTCT</td>
<td>TTTAGTGCTGCTCCACAGAG</td>
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<td>Slco2a1</td>
<td>GACAGGGTCTTTCTCTTTCCT</td>
<td>TACACCAGCTTTACTCTCTG</td>
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<tr>
<td>Tor1b</td>
<td>GGGATGCTGCTGCTCCAGTCTC</td>
<td>AGGCAACAGGGTCCAAAG</td>
</tr>
<tr>
<td>5031439G07Rik</td>
<td>TTCTCCGAGAGGACAGCAGAG</td>
<td>TTTCCATCGACTCGGTTTAG</td>
</tr>
</tbody>
</table>

Primer sequences were designed de novo utilising the Primer3 software (V0.4.0: http://bioinfo.ut.ee/primer3-0.4.0/).
3.5 Results

3.5.1 mRNA-seq data output and differentially expressed genes

Messenger RNA-sequencing (mRNA-seq) was used to identify the profile associated with the development of steatohepatitis in Hfe<sup>-/-</sup> mice. Three animals from each group of mice, Hfe<sup>-/-</sup> mice fed either chow or HCD for 20 weeks were selected as source of liver mRNA. The selected mice had 100% steatosis and fibrosis grade-1a. The liver mRNA was sequenced and the quality of the sequencing was assessed utilising the Ion Torrent Genome Suite V3.2. This assigned per-base quality scores and trimmed the adaptor sequences. It also removed reads that were shorter than 8-10 bases and those that had mixed/polyclonal reads. After filtering and trimming, between 4 and 6 million reads per sample of high quality data were obtained across all samples. All the samples had an average read quality score of 28 to 31 (Fig 3.4 A) on the Phred scale (see Appendix 3) (208) indicating a possible error rate of 1:1000. This data was then aligned to the Mus musculus reference genome - mm10, using the Torrent Mapping Alignment Programme (TMAP) aligner, based on an algorithm specific for Ion Torrent data. The mapping quality was 32 to 44 on the Phred scale indicating a possible error rate of 1:10,000. Of the post-filtered reads obtained by sequencing, an average of 95% mapped to a unique location in the genome (Fig 3.4 B, C) and a total of 23,022 genes were detected. The expression data were subjected to normalisation to reads per kilobase of exon model per million mapped reads (RPKM) (205) which normalised each sequence to the total number of reads in the sample and to the gene length. This method accounted for normalisation between and within the samples on a broad basis. Upper quartile (Q3) normalisation was also carried out to minimise the influence of highly differentially expressed genes on the RPKM normalisation. The normalised values were then subjected to logarithmic transformation (log<sub>2</sub>) to produce a normal distribution (binomial distribution of data was observed graphically) and subsequently analysed for differential expression.

This analysis provided a list of 766 genes which were found to be differentially expressed at a cut-off \( p \leq 0.05 \) and fold-change of 1.5. Of these genes, 340 were upregulated and 426 were downregulated, and have been graphically represented in a volcano plot (Fig 3.5 A). In order to streamline the analysis, more stringent filtering criteria were applied to the data. The Benjamini and Hochberg’s multiple testing criterion (209) was used to account for the false discovery rate (FDR) and utilised a \( p \) (FDR) \( \leq 0.1 \) with a fold change of \( \pm 1.5 \) which resulted in a condensed list of 124 genes.
This filtered list was utilised to perform gene ontology (GO) enrichment to assess functional clusters of genes and identify underlying biological processes. Gene ontologies that were over-represented in the upregulated gene set included pathways of fatty acid metabolism, lipid biosynthesis and storage, cellular responses related to oxidative stress, inflammation, angiogenesis and innate immunity (Table 3.3). Cellular redox homeostasis, regulation of blood coagulation, metabolic enzymatic activity and response to cytokine stimulus are some of the GO categories that are over-represented in the downregulated gene sets (Table 3.4). These gene ontologies were in agreement with changes associated with steatotic injury but did not appear to provide any additional or novel information with regards to changing biological processes in this model.

The analysis was streamlined further to enable identification of candidate genes which might be involved in disease progression therefore the most stringent filtering criteria were applied: \( p \) (FDR) \( \leq 0.05 \) with a fold change of \( \pm 1.5 \). This further reduced the list to 20 differentially expressed genes (Fig 3.5 B). Some of these genes had previously been associated with NAFLD while others had no known responses to high caloric intake or the development of steatosis. These genes formed the focus for further studies in this project and will be discussed in greater detail in this chapter.

Two genes in this list were transcript variants of the same gene (CD36) and could not be differentiated between by RT-qPCR for the purpose of further analysis. Hence this list was reduced to 19 genes.

### 3.5.2 Validation of hepatic mRNA-seq data by RT-qPCR

Validation of the 19 differentially expressed genes identified from applying the most stringent filtering criteria was performed using RT-qPCR. Liver samples from 9 mice in both groups (\( Hfe^{+/} \) mice fed HCD and \( Hfe^{+/} \) mice fed chow for 20 weeks) including the 3 mice in each group utilised for mRNA-seq were used for this purpose.

The majority of the genes followed the same pattern of gene expression as identified by mRNA-seq analysis (Fig 3.6 A). Of the 11 genes that were found upregulated in mRNA-seq, eight were validated by RT-qPCR and were found to be statistically significant at \( p \leq 0.05 \). The fold change of expression of these genes was also found to be very similar on both platforms – mRNA-seq and RT-qPCR (Fig 3.6 B). The remaining genes – 5031439G07Rik, Torsin 1b (\( Tor1b \)) and Lipase A (\( Lipa \)) – which were found to be significantly upregulated by mRNA-seq analysis were not found to be significantly upregulated by RT-qPCR.
**Fig 3.4: Pre and post alignment quality analysis of sequenced reads.** A) Quality scores were assigned for the identification of the correct nucleotide along each fragment that was sequenced and is graphically represented with the position of the base along the sequence on the X-Axis and the quality score on the Y-axis. The quality scores are above 20 up to 250 bases after which the quality scores are reduced and are fairly erratic. B) Graphical representation of the percentage of distribution of uniquely aligned reads against *Mus musculus*-mm10 genome and total number of reads per sample. All the samples had a high percentage of alignment and approximately 90% of the sequences aligned to unique location in the genome. C) Summary of sequencing statistics of the 3 control (chow fed mice) (C1-3) samples and 3 HCD (high calorie diet fed mice) samples (HCD1-3) was analysed at different stages of analysis. All samples had between above 4.8 million high quality reads of which almost 90% had aligned to a unique location in the *Mus musculus* genome.
Fig 3.5: Quantitative analysis schema of differentially expressed dataset. A) Volcano plot of mRNA-seq data. The different colours represent genes with various p-values as described in the flow chart. B) Representation of the fold change of most significantly differentially expressed genes.

Seven of the eight genes found to be downregulated by mRNA-seq were also significantly downregulated by RT-qPCR ($p \leq 0.001$). The fold change of gene expression was also very similar across the two platforms (Fig 3.6 A and B). Sequencing analysis indicated a 2.84 fold downregulation of alcohol dehydrogenase 6, pseudogene (Adh6_ps1) and this result was not validated by RT-qPCR analysis.
Overall, the changes in gene expression quantified by mRNA-seq were mostly validated by RT-qPCR. This indicates that mRNA-seq data is generally reliable but validation by RT-qPCR is imperative prior to extensive downstream analysis.

Table 3.3: Top 20 over-represented gene ontologies in upregulated gene sets with $p$ (FDR) $\leq$ 0.1 with a fold change of $\geq 1.5$.

<table>
<thead>
<tr>
<th>Over-represented gene sets of upregulated genes</th>
<th>Enrichment p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid biosynthetic process</td>
<td>6.16E-07</td>
</tr>
<tr>
<td>Lipid biosynthetic process</td>
<td>7.66E-06</td>
</tr>
<tr>
<td>Fatty acid elongation, polyunsaturated fatty acid</td>
<td>3.72E-05</td>
</tr>
<tr>
<td>Oxidation-reduction process</td>
<td>4.05E-05</td>
</tr>
<tr>
<td>Regulation of transcription in response to oxidative stress</td>
<td>9.27E-05</td>
</tr>
<tr>
<td>Positive regulation of cholesterol esterification</td>
<td>0.00013</td>
</tr>
<tr>
<td>Very long-chain fatty acid biosynthetic process</td>
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<tr>
<td>Cellular response to organic substance</td>
<td>0.000172</td>
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<tr>
<td>Long-chain fatty acid transport</td>
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</tr>
<tr>
<td>Lipid storage</td>
<td>0.000555</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>0.000611</td>
</tr>
<tr>
<td>Negative regulation of growth of symbiont in host</td>
<td>0.001035</td>
</tr>
<tr>
<td>Fatty acid metabolic process</td>
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</tr>
<tr>
<td>Negative regulation of DNA binding</td>
<td>0.001521</td>
</tr>
<tr>
<td>Negative regulation of smooth muscle cell proliferation</td>
<td>0.001945</td>
</tr>
<tr>
<td>Lipid metabolic process</td>
<td>0.002552</td>
</tr>
<tr>
<td>Innate immune response</td>
<td>0.003603</td>
</tr>
<tr>
<td>Defence response to Gram-positive bacterium</td>
<td>0.005499</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>0.005504</td>
</tr>
<tr>
<td>Regulation of blood pressure</td>
<td>0.005997</td>
</tr>
</tbody>
</table>

3.5.3 Hepatic transcriptional response in diet-induced models of steatohepatitis

Following validation of gene expression in $Hfe^{+/-}$ mice, the expression of the 14 genes which were validated by RT-qPCR was further analysed in WT control animals (28 weeks of age). Similar to the $Hfe^{+/-}$ mice, some of the WT mice fed a HCD for 20 weeks also developed NASH and had a similar percentage of steatosis accumulation as was indicated by histological analysis. These control mice however had lower ALT scores despite the development of NASH
(Fig 3.1, Table 3.1). Consistent with their genotype, these mice also had lower HICs compared to their $Hfe^{-/-}$ counterparts.

Table 3.4: Top 20 over-represented gene ontologies in downregulated gene sets with $p$ (FDR) $\leq 0.1$ with a fold change of $\geq 1.5$.

<table>
<thead>
<tr>
<th>Over-represented gene sets of downregulated genes</th>
<th>Enrichment p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidation-reduction process</td>
<td>3.03E-06</td>
</tr>
<tr>
<td>Aerobic respiration</td>
<td>0.000122365</td>
</tr>
<tr>
<td>Regulation of blood coagulation</td>
<td>0.000292037</td>
</tr>
<tr>
<td>Glycerol ether metabolic process</td>
<td>0.000628048</td>
</tr>
<tr>
<td>Cellular metabolic process</td>
<td>0.00136158</td>
</tr>
<tr>
<td>Negative regulation of endopeptidase activity</td>
<td>0.00161213</td>
</tr>
<tr>
<td>Positive regulation of phosphatidylinositol 3-kinase cascade</td>
<td>0.00255434</td>
</tr>
<tr>
<td>Negative regulation of peptidase activity</td>
<td>0.00331061</td>
</tr>
<tr>
<td>Epidermis development</td>
<td>0.00385513</td>
</tr>
<tr>
<td>Response to peptide hormone stimulus</td>
<td>0.00512867</td>
</tr>
<tr>
<td>Positive regulation of protein kinase B signaling cascade</td>
<td>0.00719102</td>
</tr>
<tr>
<td>Biosynthetic process</td>
<td>0.00885706</td>
</tr>
<tr>
<td>Response to cytokine stimulus</td>
<td>0.008857</td>
</tr>
<tr>
<td>Cell redox homeostasis</td>
<td>0.009932</td>
</tr>
<tr>
<td>Blood coagulation</td>
<td>0.013481</td>
</tr>
<tr>
<td>Regulation of proteolysis</td>
<td>0.013481</td>
</tr>
<tr>
<td>Positive regulation of cell proliferation</td>
<td>0.014942</td>
</tr>
<tr>
<td>Fatty acid metabolic process</td>
<td>0.026271</td>
</tr>
<tr>
<td>Transport</td>
<td>0.034922</td>
</tr>
<tr>
<td>Trans-membrane transport</td>
<td>0.034957</td>
</tr>
</tbody>
</table>

Gene expression was also analysed in liver tissue from WT and $Hfe^{-/-}$ mice which underwent a shorter duration (8 weeks) of dietary treatment (Fig 3.1, Table 3.1) and culled at 16 weeks of age. Mice fed chow, regardless of genotype (WT or $Hfe^{-/-}$), had normal liver histology at the end of treatment. With HCD feeding for 8 weeks the WT mice developed simple steatosis while the $Hfe^{-/-}$ mice developed steatohepatitis with early fibrosis. Gene expression was analysed across these mice as well to identify a pattern of gene expression in mice at different stages of injury namely simple steatosis versus steatohepatitis.
Adipose differentiation related protein (PLIN2) and Cell death inducing DFFA like effector c (CIDEC) are lipid droplet proteins which assist in the intracellular mobilisation and storage of proteins (Fig 3.7 A and B) (210). Expression of Plin2 was elevated 2-fold and Cidec was elevated up to 70-fold in livers of WT and Hfe<sup>−/−</sup> mice fed HCD. Gene expression of CD36, a fatty acyl translocase, was also increased in Hfe<sup>−/−</sup> mice fed HCD. Changes in expression were mainly regulated in response to HCD feeding (p ≤ 0.001). Gene expression of Cidec and CD36 was significantly increased (p ≤ 0.05) in 16 week old Hfe<sup>−/−</sup> mice which developed steatohepatitis compared to the WT controls which developed simple steatosis (Fig 3.7 C).

CyclinD1, a regulator of cell cycle progression (211) was not altered in response to a HCD in the 16 week old WT mice which developed steatosis. In contrast, Hfe<sup>−/−</sup> mice fed a HCD and developed steatohepatitis had 7-fold induction of CyclinD1 expression. This indicates an increased cell proliferation stimulus associated with the loss of HFE function and the development of a more severe phenotype (Fig 3.8 A). Aldehyde dehydrogenase 1, family L1 (Aldh1l1) and Glycosylphosphatidylinositol specific phospholipase D1 (Gpld1), both have been shown to regulate cell proliferation (212-214) and had reduced expression up to 2-fold lower (p ≤ 0.05) in response to the HCD (Fig 3.8 B and C).

Interferon, alpha-inducible protein 27 like 2B (Ifi27l2b), an interferon stimulated gene, has been shown to induce apoptosis (215) and its expression was increased with high calorie feeding only in Hfe<sup>−/−</sup> mice at both 16 and 28 weeks of age. At 28 weeks of age, WT and Hfe<sup>−/−</sup> mice fed the HCD developed steatohepatitis but a significant increase in expression of Ifi27l2b was observed only in the Hfe<sup>−/−</sup> mice (p ≤ 0.01). This increase in expression possibly indicates a direct effect of the loss of HFE function on gene expression, or an indirect effect of iron loading as a result of the Hfe knock out or the combination of loss of HFE and the HCD feeding on Ifi27l2b expression.

mRNA expression of Slco1a1, a bile acid transporter (Fig 3.9 A) (216) and Scnn1a, a sodium transporter (Fig 3.9 B) (217) was reduced in response to a HCD in WT and Hfe<sup>−/−</sup> mice (diet effect p ≤ 0.001). In the 16 week old mice, there was also a reduction of gene expression in Hfe<sup>−/−</sup> mice compared with the WT mice in mice fed chow and a HCD. This may be indicative of an effect of Hfe deletion on gene expression of Slco1a1 and Scnn1a which may be causative of the severe pathology observed in the Hfe<sup>−/−</sup> mice. Slco2a1, a prostaglandin transporter (Fig 3.9 C) (218), had reduced expression in HCD fed mice at both 16 and 28 weeks of age (diet effect p ≤ 0.01).
Fig 3.6: Genes found differentially expressed by RNA-seq were validated by RT-qPCR. A) Relative expression of up and down regulated genes were analysed in Hfe−/− mice fed chow and HCD. Data is represented as mean ± SEM N = 7-9, *p ≤ 0.05, Student’s t-test B) The fold change of gene expression for all the genes found differentially expressed was compared across the two platforms (mRNA-seq and RT-qPCR).

There was an increase in mRNA levels of Aldh3a2, an enzyme responsible for the detoxification of aldehydes (219, 220), after high calorie feeding in WT and Hfe−/− mice at both 16 and 28 weeks of age (Fig 3.10 A). At 16 weeks of age, there was also a significant increase of Aldh3a2 expression in Hfe−/− mice fed the HCD compared to the WT controls (p = 0.015). mRNA expression of Arsg, a lysosomal sulphatase, (Fig 3.10 B) (221) was also increased in mice fed a HCD (diet effect p ≤ 0.01) with an additional effect of genotype on Arsg gene expression in 16 week mice, which was increased in Hfe−/− mice compared to the WT control (p = 0.01).
Fig 3.7: Expression of genes involved in lipid storage and fatty acid uptake are increased in mice fed a HCD. Gene expression of liver tissue from WT controls and Hfe<sup>−/−</sup> mice fed either chow or a HCD at 16 and 28 weeks of age for A) Plin2 B) Cidec and C) CD36 was analysed. The results are represented as mean ± SEM. n (16wk) = 4-6, n (28wk) = 7-9 Significant effects are reported from 2-way ANOVA. Bars represent significance from a Holm-Sidak’s post-hoc test at *p ≤ 0.05. Wild type (WT), high calorie diet (HCD), adipose differentiation related protein (Plin2), cell death inducing DFFA like effector c (Cidec), fatty acyl translocase (CD36).
Hydroxysteroid dehydrogenases, *Hsd3b5* and *Hsd17b13*, belong to a group of alcohol oxidoreductases which catalyse the dehydrogenation of hydroxysteroids in the process of steroidogenesis (222, 223). At both 16 and 28 weeks of age hepatic *Hsd3b5* expression was reduced with diet (*p* ≤ 0.01) and additionally there was a reduction of expression in *Hfe*<sup>−/−</sup> mice (*p* ≤ 0.05) (Fig 3.11 A). *Hsd17b13* expression was increased in HCD fed mice (*p* ≤ 0.05) and there was an effect of genotype (*p* ≤ 0.01) at 16 weeks of age which was not present in older mice which developed steatohepatitis (Fig 3.11 B).

Finally, *Gm4956*, a predicted gene with no known function, had reduced expression in 16 week old *Hfe*<sup>−/−</sup> mice independent of the diet (*p* ≤ 0.01). With long term feeding and the development of steatohepatitis in 28 week old mice, Gm4956 mRNA expression was reduced by diet and a further reduction was observed in *Hfe*<sup>−/−</sup> mice fed a HCD (Fig 3.11 C).
3.6 Discussion

In this study, messenger RNA sequencing generated data which revealed significant changes in several genes with a broad range of functional activity. Some of these genes have been previously associated with NAFLD, but other genes which were found to be differentially expressed have not been associated with liver disease or high calorie feeding and may have an unrecognised role in the development of liver injury. The most notable gene expression changes in the steatotic liver with up to 70-fold increase was Cidec (Cell death inducing DFFA like effector c) followed by Ifi27l2b (Interferon, alpha-inducible protein 27 like 2B) with a 5-fold increase in expression. Hsd3b5 (3-β-Hydroxysteroid dehydrogenase Type 5) had the biggest fold downregulation (13-fold) in steatotic livers.

The most significantly altered genes found from sequencing analysis were categorised on the basis of function of their transcribed proteins and indicate a dynamic liver microenvironment of adaption to lipid accumulation and pro-inflammatory stimuli. CIDEC and PLIN2 mediate intracellular storage of triglycerides (210) and CD36 facilitates uptake of fatty acids into the liver (224). As would be expected, expression of all three genes transcribing these proteins was increased with the development of steatosis. Other genes found to be differentially expressed have an established or a putative role in cell proliferation and apoptosis. CyclinD1, Aldh1l1 and Gpld1 had expression patterns consistent with an increase in proliferative capacity with HCD feeding (211, 213, 214, 225). Ifi27l2b on the other hand, an interferon stimulated gene with a role in promoting apoptosis (215), was upregulated in Hfe−/− mice fed a HCD and suggested an increase in apoptosis stimulus associated with the development of steatohepatitis

Membrane transporters SLCO1A1, SLCO2A1 and SCNN1A, have a role in bile acid (216), prostaglandin (218) and sodium transport (217, 226) respectively, and the genes which transcribe these proteins were all downregulated with high calorie feeding. Aldh3a2, an aldehyde dehydrogenase with a role in detoxification of compounds arising from lipid peroxidation (219, 220) and Arsg responsible for heparan sulphate degradation (221) were both upregulated. Hydroxysteroid dehydrogenases, Hsd3b5 and Hsd17b13, involved in steroid inactivation (222, 223) and lastly, a predicted gene GM4956, with no known function were also differentially regulated.
Fig 3.8: Cell proliferation and apoptosis stimulus was increased in mice fed a HCD. Gene expression of liver tissue from WT controls and $Hfe^{+/-}$ mice fed either chow or a HCD at 16 and 28 weeks of age for A) CyclinD1 B) Aldh1l1 C) Gpld1 and D) Ifi27l2b was analysed. The results are represented as mean ± SEM. n (16wk) = 4-6, n (28wk) = 7-9. Significant effects are reported from 2-way ANOVA. The bars represent significance from a Holm-Sidak’s post-hoc test at *$p \leq 0.01$. Wild type (WT), high calorie diet (HCD), aldehyde dehydrogenase 1, family L1 ($Aldh1l1$), glycosylphosphatidylinositol specific phospholipase D1 ($Gpld1$), interferon, alpha-inducible protein 27 like 2B ($Ifi27l2b$).
Fig 3.9: Gene expression of membrane transporters was reduced in mice fed a HCD. Gene expression of liver tissue from WT controls and Hfe<sup>−/−</sup> mice fed either chow or a HCD at 16 and 28 weeks of age for A) Slco1a1, B) Scnn1a, and C) Slco2a1 was analysed. The results are represented as mean ± SEM. n (16wk) = 4-6, n (28wk) = 7-9. Significant effects reported from 2-way ANOVA. The bars represent significance from a Holm-Sidak’s post-hoc test at *p ≤ 0.05. Wild type (WT), high calorie diet (HCD), solute carrier organic anion transporter family 1a1 (Slco1a1), sodium channel, non-voltage-gated 1 alpha subunit (Scnn1a), solute carrier organic anion transporter family member 2a1 (Slco2a1).
Fig 3.10: Expression of genes involved in degradation of aldehydes and heparan sulphates respectively was increased in mice fed a HCD. Gene expression of liver tissue from WT controls and \( Hfe^{−/−} \) mice fed either chow or a HCD at 16 and 28 weeks of age for A) \( Aldh3a2 \) and B) \( Arsg \) was analysed. The results are represented as mean ± SEM. \( n \) (16wk) = 4-6, \( n \) (28wk) = 7-9. Significant effects are reported from 2-way ANOVA. The bars represent significance from a Holm-Sidak’s post-hoc test at \(*p \leq 0.01\). Wild type (WT), high calorie diet (HCD), aldehyde dehydrogenase family 3, subfamily A2 (\( Aldh3a2 \)), arylsulfatase G (\( Arsg \)).

Of the genes found differentially expressed, some had particularly interesting functions and have been further described below.

**Interferon, alpha-inducible protein 27 like 2B (\( Ifi27l2b \), upregulated in \( Hfe^{−/−} \) mice fed HCD)** belongs to a family of small interferon-alpha (IFN\( \alpha \)) inducible genes. \( Ifi27l2b \) encodes a hydrophobic protein that is located on the inner mitochondrial membrane and overexpression of \( Ifi27l2b \) has led to mitochondrial membrane depolarisation, release of cytochrome C and subsequent mitochondria mediated apoptosis (215). Increased expression of \( Ifi27l2b \) has been observed in response to stimulation with IFN\( \alpha \) and a toll like receptor stimulant (poly I:C) and
overexpression has led to caspase-dependent cell death (215). The human equivalent of this gene IFI27 was found strongly induced by IFNα and poly I:C, and had 3-17 fold higher expression in primary human keratinocytes on treatment with TNFα, IFNγ and TGFβ1 (227, 228). Consistently, Ifi27l2b was upregulated with HCD feeding only in the Hfe<sup>−/−</sup> mice in which there is a more pronounced inflammatory environment.

**Fig 3.11: Gene expression of Hydroxysteroid dehydrogenases and Predicted gene 4956.** Gene expression of liver tissue from WT controls and Hfe<sup>−/−</sup> mice fed either chow or a HCD at 16 and 28 weeks of age for A) Hsd3b5 B) Hsd17b13 and C) Gm4956 was analysed. The results are represented as mean ± SEM. n (16wk) = 4-6, n (28wk) = 7-9. Significant effects are reported from 2-way ANOVA. The bars represent significance from a Holm-Sidak’s post-hoc test at *p ≤ 0.05. Wild type (WT), high calorie diet (HCD), 3-β-hydroxysteroid dehydrogenase Type 5 (Hsd3b5), hydroxysteroid (17-Beta) dehydrogenase 13 (Hsd17b13), predicted gene 4956 (Gm4956).
**Arylsulfatase G (Arsg, upregulated in Hfe<sup>-/-</sup> mice fed HCD)** is a lysosomal sulphatase with a critical role in heparan sulphate degradation. Impairment of arylsulphatases has led to severe neuropathologies because of greatly increased storage of glycosaminoglycans (predominantly heparan sulphate) (221). ARSG acts on the N-sulfoglucosamine-3-O-sulphate (GlcNS3S) moiety during heparan sulphate degradation and consequently Arsg knockout mice have shown to have approximately 10-fold increase in heparan sulphate accumulation in the liver compared with WT mice (221). Heparan sulphate is a glycosaminoglycan (GAG) which is ubiquitously expressed in mammalian tissues in the form of a proteoglycan and is involved in many different stages of inflammation (229) and increased abundance and altered localisation of heparan sulphate has been associated with fibrogenic liver diseases and hepatocellular cancer (230). There is no observed role for ARSG activity in fatty liver disease but Arsg<sup>-/-</sup> mice had secondary lipid and cholesterol accumulation in the cerebellum indicating a potential role for Arsg in lipid metabolism (231). The increase of Arsg expression in mice with steatohepatitis could indicate a role for heparan sulphate accumulation in its development.

**Glycosylphosphatidylinositol phospholipase D1 (Gpld1, downregulated with HCD in WT and Hfe<sup>-/-</sup> mice)** has a function in cleavage of GPI-anchored proteins and their subsequent release from cellular membranes (232). Gpld1 is highly expressed in the liver and is known to associate with triglyceride-rich lipoproteins under various dietary and pathological conditions (233). Increased serum levels and a 3-fold increase of mRNA expression of Gpld1 have also been observed in NAFLD patients (234). Conversely, reduced expression of Gpld1 has been observed in patients with hepatocellular carcinoma (214).

Two independent research groups have investigated the role of overexpression of Gpld1 in HepG2 cells (human hepatoma cell line). One study performed expression analysis on the overexpressing cells and observed increased expression of *de novo* lipogenesis genes (*Scd1, Dgat1, Acc2*) (234), while the other study observed a reduced proliferative capacity of cells overexpressing Gpld1 (214). In this study, reduced Gpld1 expression has been observed in mice following high calorie feeding and could possibly indicate a process adverse to *de novo* lipogenesis in these mice and an increased proliferative capacity.

**Cluster of differentiation (CD36, upregulated in Hfe<sup>-/-</sup> mice fed HCD)** also called fatty acyl translocase (FAT) is a membrane glycoprotein that belongs to the class B scavenger receptor family with a primary role in facilitating uptake of fatty acids into hepatocytes. The basal level of CD36 in hepatocytes is low but has been shown to increase significantly with high fat
feeding and in obese mice (235-237). CD36 expression was also increased in NAFLD cohorts and was positively correlated with fat content (238). CD36 is versatile in its biological roles and one other function which is thought to be important in injury manifestation is its pro-apoptotic properties (239). Another study has observed an elevation of CD36 expression in NASH patients and gene expression was positively correlated with TUNEL positive cells (240). MitoNEET, a mitochondrial protein, has been characterised as a molecule with two Fe-S clusters and acts as a regulator of mitochondrial iron content (241, 242). MitoNEET in adipose tissue has been implicated in enhancing CD36 expression (99) and this potentially provides a mechanistic link for fat accumulation and disrupted iron concentrations as seen in Hfe<sup>-/-</sup> mice. Consistent with this, CD36 gene expression was also found significantly higher in Hfe<sup>-/-</sup> mice fed a HCD which developed steatohepatitis compared to WT mice fed HCD which developed simple steatosis. This MitoNEET and CD36 link requires further investigation in the liver and in this model of steatohepatitis to delineate this mechanism.

**Solute carrier organic anion transporter family member 1a1 (Slco1a1/Oatp1, downregulated in Hfe<sup>-/-</sup> mice fed HCD)** belongs to a family of transmembrane transporters and mediates the uptake of conjugated and unconjugated bile acids into the liver (216). Expression of this gene was found to be suppressed in a methionine choline deficient diet (MCD) induced model of steatohepatitis in association with increased serum bile acids and expression of pro-inflammatory cytokines (243). In a model of infectious colitis, Slco1a1 downregulation was abrogated in IL6 null mice indicating a role for inflammatory cytokines in transporter regulation (244).

**Sodium Channel, Non-Voltage-Gated 1 Alpha Subunit (Scnn1a, downregulated in Hfe<sup>-/-</sup> mice fed HCD)** gene encodes the alpha subunit of the non-voltage gated epithelial sodium channel (αENaC). There is no previously reported evidence for expression of this gene in the liver but dysregulated αENAC in the kidney has been associated with liver cirrhosis (245). Expression of this gene was found to be suppressed with hydrogen peroxide induced oxidative stress and by pro-inflammatory cytokine TGF-β1 (246, 247).

In this study, Slco1a1 and Scnn1a expression was suppressed following a HCD and there was a further reduction in gene expression associated with the lack of functional HFE. Both these genes had a similar trend of gene expression which reduced in WT mice fed a HCD and a further reduction in Hfe<sup>-/-</sup> mice fed a HCD. Moreover, both these genes have been suppressed by TGF-β1 (243, 247, 248). Given the differential expression in Hfe<sup>-/-</sup> mice with the loss of
HFE function – a major histocompatibility complex, one can speculate a role for an altered inflammatory response in altered membrane transporters in steatohepatitis.

3.7 Summary and conclusion

A transcriptomics approach has been utilised to identify novel genes and processes underlying the development of injury in a diet induced model of steatohepatitis with an added insult of iron loading as a result of the Hfe gene mutation. Some of the genes identified as differentially expressed appear to be altered by the loss of HFE function and might have a role in exacerbating steatohepatitis pathology.

The observed gene expression changes might be a result of one of many primary factors including HCD-induced steatosis, the knockout of the Hfe gene itself, the resultant iron overload or a combination of these factors. Alternatively, gene expression changes could be a result of the development of oxidative stress, inflammatory cytokine release and insulin resistance which are important factors associated with the development of steatohepatitis injury (91, 97). Therefore I hypothesized that the differential expression of these genes was directly or indirectly involved in the molecular pathology of Hfe-associated steatohepatitis.

The specific role of these differentially expressed genes was yet to be understood and subsequent work in this thesis was aimed at further examining these genes in different models of chronic liver disease. Furthermore, a model of fat and iron loading in vitro was developed to enable the investigation of gene expression changes in isolated hepatocytes devoid of systemic and endocrine effects associated with in vivo analyses. This work was also extended to modify expression of candidate genes in vitro to examine the subsequent downstream effects on lipid and iron metabolism, inflammation and fibrogenic mechanisms.
Chapter 4  *Expression Analysis and Mechanisms of Pathogenesis of Arsg, Gpld1 and Ifi27l2b in Chronic Liver Injury*
4.1 Introduction

Hepatic transcriptomics analysis, in the previous chapter, has identified genes with previously unrecognised roles in the development of steatohepatitis and expression analysis of these genes was investigated in livers with normal pathology, steatosis and steatohepatitis. Most of the genes found to be differentially regulated were largely altered in response to a high caloric intake. Three genes however were of particular interest and have formed the focus of further work in this project. *Ifi27l2b* and *Arsg* were identified as genes which were regulated in part by genotype (*Hfe*<sup>−/−</sup>) and had more pronounced differential expression with the development of steatohepatitis, and neither of these genes has been previously identified in the development of liver injury. *Gpld1*, on the other hand is known to be associated with high-density lipoproteins and has been upregulated in NAFLD (234). This gene was of particular interest given the contradictory result in this thesis which has found downregulation of *Gpld1* in livers of mice with steatohepatitis. With these genes as the main focus, the primary goal of this study was to examine the expression of *Ifi27l2b*, *Arsg* and *Gpld1* in other models of chronic liver disease in order to determine if the observed alterations in gene expression were specifically associated with this model of NASH or rather a generalised response to the development of chronic liver injury.

In order to address this aim, murine models of alcoholic steatohepatitis and fibrosis have been investigated in this study. ASH is a liver disease that is generally histologically indistinguishable from NASH. Although these two conditions vary in the primary insult for disease development the pathogenic mechanisms converge on altered lipid metabolism and the accumulation and deposition of triglycerides in lipid droplets. ASH and NASH also have many similarities in pathogenesis including, oxidative stress and pro-inflammatory stimuli which can ultimately progress to fibrosis and eventually cirrhosis, which represents a common end-point for both these chronic liver diseases (249). A model of concomitant high calorie and/or excessive alcohol intake in *Hfe*<sup>−/−</sup> mice, which resulted in non-alcoholic and alcoholic steatohepatitis respectively, has been used for this study (10, 250). These animals were provided 20 % (vol/vol) ethanol in drinking water for the same duration (8 weeks) as the feeding of HCD (250) and were investigated as part of an independent study for which the mice were characterised and the liver and serum parameters are represented in Fig 4.1(250). The mice fed HCD alone, developed NASH, these mice had 95 % steatosis with increased serum ALT and developed early fibrosis. The mice fed alcohol on the other hand irrespective of their diet developed ASH. These mice had lower steatosis compared to mice with NASH and also
had lower serum ALT. ASH mice however, had a higher fibrosis score compared to mice with NASH (Fig 4.1).

On the other hand, the *Mdr2*−/− mouse is the genetic equivalent of progressive familial intrahepatic cholestasis type 3 (PFIC3) mutation in humans and spontaneously develops severe fibrotic injury induced by leaked bile acids into the portal tracts of the liver (251). This leakage induces a severe inflammatory phenotype and activation of hepatic stellate cells in the *Mdr2*−/− mice (252, 253). Injury in this model is devoid of lipid droplet formation and the development of steatosis and histological parameters are outlined in Fig 4.2. This model has been investigated at three time points, at the beginning of fibrosis at 3 weeks of age, the peak of pro-inflammatory and pro-fibrogenic markers at 8 weeks of age and the subsequent plateau of this phenotype at 12 weeks of age to assess gene expression at different stages of liver injury.

Additionally this chapter has assessed the downstream factors associated with previously identified functions of *Arsg*, *Ifi27l2b* and *Gpld1* to describe a role of these genes in this model of NASH and in a model of hepatic fibrotic injury with severe inflammation and devoid of lipid accumulation.

### 4.2 Hypothesis

The changes in gene expression observed are not only altered in NASH in response to iron and fat loading but are generalised responses to liver injury independent of the primary insult.

### 4.3 Aims

The specific aims were:

1. To validate protein expression of ARSG, GPLD1 and IFI27L2B in liver of WT and *Hfe*−/− mice fed chow and HCD.
2. To assess gene expression of *Arsg*, *Gpld1* and *Ifi27l2b* in murine models of alcoholic steatohepatitis and fibrosis.
3. To examine downstream factors associated with differential gene expression to identify potential mechanisms of pathogenicity.
Fig 4.1: Liver histology of Hfe<sup>-/-</sup> mice fed chow or a HCD in the absence and presence of alcohol consumption. A) Representative images (at 20X magnification) of haematoxylin and eosin stained liver sections are presented with different grades of steatosis. B) Histological diagnosis, serum parameters and hepatic iron concentration have been described previously (10, 250). Fibrosis stages described are: 1 = perivenular, perisinusoidal and pericellular fibrosis, 2 = focal or extensive periportal fibrosis, 3 = bridging fibrosis, focal or extensive and 4 = Cirrhosis. Data is presented as median (range) or mean ± SD. *p ≤ 0.05 compared with mice fed the same diet and no alcohol consumption (10, 250). High calorie diet (HCD), ethanol (EtOH), alanine transaminase (ALT), hepatic iron concentration (HIC).
**Fig 4.2: Liver histology of Mdr2<sup>+/+</sup> (WT) and Mdr2<sup>-/-</sup> mice at 3, 8 and 12 weeks of age.**  
A) Representative images (at 20X magnification) of Sirius-red stained liver sections. B) Histological scoring and serum alanine transaminase (ALT). The data is presented as median (range) and mean ± SEM. N = 4-8 *p ≤ 0.05 Mdr2<sup>+/+</sup> vs Mdr2<sup>-/-</sup> of the same age (unpublished data).

<table>
<thead>
<tr>
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<th>Mdr2&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mdr2&lt;sup&gt;-/-&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3wk</td>
<td>8wk</td>
</tr>
<tr>
<td>% Steatosis</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Fibrosis stage</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>ALT (u/l)</td>
<td>49 ± 22</td>
<td>35 ± 5</td>
</tr>
</tbody>
</table>
4.4 Materials and Methods

4.4.1 Real time – Quantitative PCR
Gene expression analysis was performed by real time quantitative PCR as described in Chapter 2, section 2.6. Gene expression of all genes was normalised to Gapdh, B2mg and Btf3.

4.4.2 Western Blot
Western blot analysis for ARSG, GPLD1, IFI27L2B and Syndecan-1 (SDC1) was performed as described in Chapter 2, Section 2.9. GAPDH protein was used as the loading control to normalise protein expression.

Preparation of serum samples – Serum samples were diluted 1:50 in 1X Tris-buffered saline (TBS) and 7 μl of the diluted serum was mixed with 2 μl loading buffer (0.625 M Tris pH 6.8, 50 % Glycerol, 10 % sodium dodecyl sulphate, 500 mM DTT and 0.25 % bromophenol blue). Samples were not heated and loaded directly on a gel. The gel was run as described previously in Chapter 2, Section 2.9. One control sample was run on all membranes as a calibrator. Serum protein was normalised to the band intensity of this calibrator respectively for each membrane.

4.4.3 TUNEL staining
Paraffin-embedded liver sections were dewaxed in xylene (3 times for 5 min each) and hydrated in graded ethanol starting with three 30 s washes with 100 % ethanol (EtOH), followed by 30 s washes in 90 %, 70 % and 50 % of EtOH respectively. Antigen retrieval of the dewaxed liver sections was then performed using Proteinase K (2.15 μg/ml) for 10 min at room temperature (RT). Following 2 washes in de-ionised (DI) water, inactivation of endogenous peroxidases was performed by incubation of sections in 3 % hydrogen peroxide (prepared in 1X PBS) solution for 5 min. As a positive control for terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining, one section was pre-treated with DNase1 buffer for 5 min followed by treatment with DNase1 for 10 min. The negative control was treated with the buffer alone. The liver sections were washed twice in 1X phosphate buffered saline (PBS). An equilibration buffer was applied to sections for 10 min at RT and was followed by application of terminal deoxynucleotidyl transferase (TdT) enzyme (77 μl of reaction buffer + 33 μl of TdT stock) for 1 hr at 37 °C in a humidified chamber. The enzymatic reaction was stopped using a STOP buffer (100 μl STOP buffer + 3.4 ml DI water) for 10 min. The liver sections were washed twice in 1X PBS. Anti-deoxygenin conjugate was applied for 30 min in the dark at RT. The sections were washed 4 times in 1X PBS. Diaminobenzidine (DAB) was prepared in the provided buffer and the substrate
was developed for 5 min at RT. Finally, slides were counterstained in Mayer’s haematoxylin for 5 min followed by rinsing in running tap water. Slides were dehydrated through incubation with 90 % EtOH for 2 min and twice in 100 % EtOH for 2 min each and, cleared in xylene and mounted in Depex.

4.4.4 Statistical analysis

Relative expression data from RT-qPCR analysis and protein expression data from western blot analysis was log transformed: \( \log_{10}(x) + 1 \), to transform the data into a normal distribution. The log transformed data was subjected to a 2-way analysis of variance (ANOVA). The effects of the respective treatments at \( p \leq 0.05 \) were considered significant and have been reported. In experiments where an interaction of the respective treatments was found significant, the individual effects are not reported. In this case, Holm-Sidak’s post-hoc test was performed and the differences between individual groups are represented.

All statistical analysis was performed using the IBM SPSS statistics v22 (IBM Corp, Armonk, NY, USA) and graphs were generated using GraphPad prism v6.0 (La Jolla, California, USA).
4.5 Results

4.5.1 Arylsulfatase G and heparan sulphate proteoglycans

ARSG protein was increased with HCD in both WT and $Hfe^{−/−}$ mice but these changes were not significant (Fig 4.3 A). Arsg expression appeared to be induced with HCD and alcohol feeding alone but with concomitant administration of HCD and alcohol Arsg expression remained unchanged in comparison with chow fed mouse livers (Fig 4.3 B). These changes were not significant and the interaction of gene expression with alcohol and HCD feeding approached significance ($p = 0.06$). Arsg mRNA expression changes were not significant in fibrotic livers (Fig 4.3 C) but protein expression was reduced significantly with an overall effect of age ($p \leq 0.001$) and genotype ($p \leq 0.01$) (Fig 4.3 D).

ARSG, is an heparan sulphate (HS) degrading enzyme and it was hypothesised that increased hepatic ARSG would result in reduction of heparan sulphate proteoglycan (HSPG). Expression of Syndecan-1 (SDC1), the most predominant HSPG in the liver, was analysed and contrary to this hypothesis, protein analysis showed an increase in hepatic SDC1 in WT mice fed a HCD (Fig 4.4 A). Consistently, serum SDC1 was increased ($p \leq 0.01$) in WT mice fed a HCD (Fig 4.4 B).

4.5.2 Hepatic Gpld1 expression is reduced in NASH, ASH and fibrosis

GPLD1 protein was unchanged in WT livers which developed steatohepatitis. In $Hfe^{−/−}$ mice fed a HCD however, significantly lower levels ($p \leq 0.01$) of GPLD1 were observed (Fig 4.5 A). This reduction after HCD feeding is consistent with the observed levels in mRNA expression (Chapter 3, Fig 3.8). GPLD1 is a soluble protein, and the liver is the primary organ which contributes to GPLD1 in the serum therefore, serum GPLD1 was examined. Serum GPLD1 was found to be reduced in $Hfe^{−/−}$ mice fed a HCD, a similar observation to the protein expression in the liver (although the observed change was not statistically significant) (Fig 4.5 B).

Previous studies have observed elevated hepatic gene expression and serum levels of GPLD1 in NAFLD patients (234) and high levels of serum GPLD1 have been associated with high fat and fructose diets (233). Conversely, this study has shown reduced Gpld1 mRNA with HCD feeding and development of NASH and a consistent reduction in hepatic and serum GPLD1 protein.
**Fig 4.3: Hepatic Arsg expression in NASH, ASH and fibrosis.** A) Hepatic Arsg relative intensity was examined in WT and Hfe\(^-\) mice fed chow and HCD (n = 7-9) by western blot analysis and normalised to expression of GAPDH. B) Arsg relative expression was analysed in Hfe\(^-\) mice fed chow or HCD in the presence and absence of alcohol (n = 4-6) and in C) WT and Mdr2\(^-\) mice at 3, 8 and 12 weeks of age (n = 6-9). D) ARSG relative protein expression in WT and Mdr2\(^-\) mice at 3, 8 and 12 weeks of age (four animals in each group) was quantified and an image of a representative western blot is presented. In the graphs the term relative expression denotes gene expression while relative intensity denotes protein expression. The results are represented as mean ± SEM. The significant effects are reported from 2-way ANOVA. High calorie diet (HCD), wild type (WT), Mdr2\(^-\) knock out (KO).
Fig 4.4: Hepatic and serum Syndecan-1 (SDC1) increased in WT mice fed HCD. A) Hepatic SDC1 western analysis yielded several non-specific bands but serum analysis yielded only one band at 75kDa hence this band was quantified in both western blots. SDC-1 relative intensity was examined in WT and Hfe−/− mice fed chow and HCD (n = 7–9) by western blot analysis and normalised to expression of GAPDH. B) Serum SDC1 expression was also quantified across three separate membranes to accommodate all samples (biological replicates) by western blot and a calibrator sample (red box) was loaded on all gels and band intensity was normalised to this sample. The results are represented as mean ± SEM. The bars represent significance from Holm-Sidak’s post-hoc test at *p ≤ 0.001. High calorie diet (HCD), wild type (WT).

Gpld1 gene expression was reduced with HCD feeding and there was a further reduction with alcohol feeding in both mice fed chow and HCD and the development of ASH (Fig 4.6 A). The overall effect of alcohol on the reduction of gene expression was statistically significant (p ≤ 0.01). In the Mdr2−/− mice, there was a reduction of expression only in 8 week KO mice (p ≤ 0.05) but this change was not observed with the GPLD1 protein (Fig 4.6 B).
**Fig 4.5: Hepatic and serum GPLD1 is reduced in Hfe<sup>−/−</sup> mice fed HCD.** A) Hepatic GPLD1 relative intensity was examined in WT and Hfe<sup>−/−</sup> mice fed chow and HCD for 20 weeks (n = 7-9) by western blot analysis and normalised to expression of GAPDH. B) Serum GPLD1 expression was also quantified across three separate membranes to accommodate all samples (biological replicates) by western blot and a calibrator sample (red box) was loaded on all gels and band intensity was normalised to this sample. While there were a few non-specific bands in the serum western blot for GPLD1, the brightest band at 93 kDa (expected molecular weight) was quantified. The results are represented as mean ± SEM. The bars represent significance from Holm-Sidak’s post-hoc test at p ≤ 0.05. Glycosylphosphatidylinositol phospholipase D1 (GPLD1), high calorie diet (HCD), wild type (WT).

**4.5.3 Increased expression of Ifi27l2b does not correspond to increased apoptosis in liver tissue**

*Ifi27l2b* is an interferon stimulated gene and its overexpression has induced apoptosis in a mouse tumour cell line (215). Hepatic *Ifi27l2b* gene expression was found significantly increased in *Hfe<sup>−/−</sup>* mice fed a HCD and consistent with the results of gene expression studies, an increase in IFI27L2B protein was also observed in these livers (Fig 4.7 A). Given the literature on IFI27L2B as a pro-apoptotic protein, TUNEL analysis was performed on formalin-fixed liver sections to assess the presence of apoptotic nuclei. In both WT and *Hfe<sup>−/−</sup>* mice, very
few TUNEL positive cells were observed with no detectable changes with HCD feeding either (Fig 4.7 B).

**Fig 4.6: Hepatic Gpld1 expression is reduced in ASH and at the peak of fibrosis.** A) Gpld1 relative expression was analysed in Hfe<sup>−/−</sup> mice fed chow or HCD in the presence and absence of alcohol (n = 4-6) (Left) and in WT and Mdr2<sup>−/−</sup> mice at 3, 8 and 12 weeks of age (n = 6-9) (Right). B) GPLD1 relative protein expression in WT and Mdr2<sup>−/−</sup> mice at 3, 8 and 12 weeks of age (four animals in each group) was quantified and an image of a representative western blot is presented. The results are represented as mean ± SEM. In the graphs the term relative expression denotes gene expression while relative intensity denotes protein expression. The significant effects are reported from 2-way ANOVA and the bar represents significance from Holm-Sidak’s post-hoc test at *p ≤ 0.01. High calorie diet (HCD), wild type (WT), Mdr2<sup>−/−</sup> knock out (KO).
Unlike in NASH livers there was no increase in Ifi27l2b expression in ASH livers (Fig 4.8 A). In the Mdr2+ mice, a model of liver fibrosis, Ifi27l2b expression was significantly increased with the development of fibrosis. In livers of 3 week old Mdr2+ mice, with signs of early fibrosis, a 7-fold higher expression was observed. With increasing severity of fibrosis at 8 and 12 weeks of age, Ifi27l2b expression of up to 60-fold higher was observed in the Mdr2+ mice (p ≤ 0.001 at all ages) compared to the age matched WT mice (Fig 4.8 B). This increase was also evident at the protein level (p ≤ 0.001) (Fig 4.8 C). Subsequent TUNEL analysis of parrafin-fixed liver tissue detected TUNEL positive cells which were limited to the inflammatory cells (Fig 4.8 D). The observed proportion of TUNEL positive cells was however not consistent with the stark overexpression of pro-apoptotic, IFI27L2B.

Given the absence of TUNEL positive nuclei, mitochondrial genes were analysed to determine if the increased expression of Ifi27l2b, a mitochondria localised protein, indicated an increase in mitochondrial function and biogenesis. To test this hypothesis expression of Nuclear Respiratory Factor 1 (Nrf1), a transcription factor required for mitochondrial DNA transcription and Cytochrome oxidase IV (CoxIV), a mitochondrial respiratory chain complex were analysed. Nrf1 was reduced with HCD feeding in WT and Hfe+ mice and CoxIV expression remained unchanged and did not reflect increased mitochondria biogenesis. Expression of Nrf1 and CoxIV were both significantly increased (p ≤ 0.001) but only in 8 week old Mdr2+ mice, the age at which fibrogenesis was at its peak. While this result is of interest, it did not explain the significant increase of IFI27L2B at all stages of fibrosis development (Fig 4.9 A and B).
Fig 4.7: Increased expression of pro-apoptotic protein IFI27L2B is not associated with apoptosis. A) Hepatic IFI27L2B protein expression from WT and Hfe<sup>-/-</sup> mice fed chow and HCD (n = 7-9) for 28 weeks was examined by western blot analysis and normalised to expression of GAPDH. B) TUNEL assay was performed on liver section of WT and Hfe<sup>-/-</sup> mice fed chow and HCD for 28 weeks mice and representative images are presented with images of the positive and negative control. The results are represented as mean ± SEM. No significant interaction was identified from 2-way ANOVA. Interferon alpha-inducible protein 27 like 2b (IFI27L2B), high calorie diet (HCD), wild type (WT).
Fig 4.8: Hepatic Ifi27l2b expression is increased in NASH and fibrosis. A) Relative mRNA expression of Ifi27l2b was analysed in Hfe<sup>−/−</sup> mice fed chow or HCD in the presence and absence of alcohol (n = 4-6) and in B) WT and Mdr2<sup>−/−</sup> mice at 3, 8 and 12 weeks of age (n = 6-9). C) IFI27L2B relative protein expression in WT and Mdr2<sup>−/−</sup> mice at 3, 8 and 12 weeks of age (four animals in each group) was quantified and an image of a representative western blot is presented. D) Representative images (at 20X magnification) of WT and Mdr2<sup>−/−</sup> formalin-fixed liver sections stained for TUNEL positive nuclei which are indicated by the arrows. In the graphs the term relative expression denotes gene expression while relative intensity denotes protein expression. The results are represented as mean ± SEM. The bars represent significance from Holm-Sidak’s post-hoc test at *p ≤ 0.01. High calorie diet (HCD), wild type (WT), Mdr2<sup>−/−</sup> knockout (KO).
Mitochondria biogenesis was not increased in mice with increased expression of Ifi27l2b. WT and Hfe\textsuperscript{+/−} mice fed chow and HCD (n = 7-9) (left) and WT and Mdr2\textsuperscript{+/−} mice at 3, 8 and 12 weeks of age (n = 6-9) (right) were assessed for changes in A) Nrf1 and B) CoxIV. The results are represented as mean ± SEM. The significant effects are reported from 2-way ANOVA and the bars represent significance from Holm-Sidak’s post-hoc test at *p ≤ 0.01. High calorie diet (HCD), wild type (WT), Mdr2\textsuperscript{+/−} knockout (KO).
4.6 Discussion

The primary aim of this study was to investigate the expression of 3 candidate genes - Arsg, Gpld1 and Ifi27l2b in ASH, a liver disease with histological similarity to NASH, and in the Mdr2⁻/⁻ murine model of fibrosis, to examine a common end-point of progressive liver disease. Overall findings from this study suggest that changes in gene expression of these three genes are not only seen in NASH but also occur in other models of liver injury. The results from this study have been summarised in Table 4.1 and have been discussed below.

Table 4.1: Summary of expression analysis of Arsg, Gpld1 and Ifi27l2b in mouse models of NASH, ASH and fibrosis.

<table>
<thead>
<tr>
<th></th>
<th>NASH</th>
<th>ASH</th>
<th>Mdr2⁻/⁻ (8 wk)</th>
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<tbody>
<tr>
<td></td>
<td>RNA</td>
<td>Protein</td>
<td>RNA</td>
</tr>
<tr>
<td>Arsg</td>
<td>↑*</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Gpld1</td>
<td>↓</td>
<td>↓*</td>
<td>↓</td>
</tr>
<tr>
<td>Ifi27l2b</td>
<td>↑↑*</td>
<td>↑↑</td>
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</table>

RNA and protein expression of Arsg, Gpld1 and Ifi27l2b was analysed in NASH, ASH and fibrotic livers. Red arrows indicate upregulation and green arrows indicate downregulation in comparison to the control mice with normal liver histology. One arrow ≤ 2 fold change, two arrows ≥ 2 fold change, four arrows ≥ 50 fold change. The * represents significance from Holm-Sidak’s post-hoc test at p ≤ 0.05. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b), non alcoholic steatohepatitis (NASH), alcoholic steatohepatitis (ASH).

ARSG was upregulated with HCD feeding and downregulated in Mdr2⁻/⁻ mice and indicates that it is differentially modulated in conditions devoid of lipid loading. ARSG has been recently characterised as a critical enzyme for HS degradation and knock out of this gene has led to development of a lysosomal storage disease with accumulation of enlarged, vacuolated lysosomes and accumulation of heparan sulphate in the liver (221, 231). It was hypothesized that Arsg degrades heparan sulphate aggregates to affect plasma clearance of triglycerides and hence ameliorates hepatic lipid accumulation. Additionally, Syndecan-1 the most predominant HSPG in the liver and has a putative role in mediating hepatic clearance of triglyceride-rich lipoproteins (254). Contrary to the expected reduction of Syndecan-1, hepatic and serum protein was increased in WT HCD fed mice. This observation is consistent with previous reports that serum syndecan-1 has increased in patients with NALFD (255). Several enzymes
with a role in heparan sulphate biosynthesis, sulphation and degradation exist, altered expression of which have been associated with various pathogenic conditions. While Arsg expression was not consistent with changes in Syndecan-1, there are several other heparan sulphate proteoglycans which might be affected by ARSG expression and need to be investigated.

A reduction in hepatic *Gpld1* in *Hfe*⁻⁻ mice with NASH has been observed with a further reduction in mice with ASH. Furthermore, in *Mdr2*⁻⁻ mice which do not develop steatosis no significant changes were observed. Given this data and reports from the literature on *Gpld1* expression, it is tempting to speculate that *Gpld1* expression is transcriptionally regulated by changes associated with lipid accumulation. Similar to the observation in this study, reduced expression of *Gpld1* in the liver and serum has also been observed in patients with HCC (214). On the contrary, a previous study in NAFLD patients has shown increased expression of hepatic and serum GPLD1 (234). This contradictory observation could possibly indicate a shift to a proliferative phenotype and the development of HCC (214). Given this switch in gene expression from overexpression in NAFLD to downregulation in HCC, it might be pertinent to investigate the use of serum GPLD1 as a potential biomarker for progressive liver disease. It is however, essential to investigate underlying mechanisms that govern *Gpld1* expression to better interpret the observed serum parameters.

This study has demonstrated a pronounced increase in *Ifi27l2b* in mice with NASH and at all stages of fibrosis. This interferon stimulated gene, with a pro-apoptotic role was not associated with apoptosis in either of the models in which overexpression of IFI27L2B was observed. IFI27L2B is known to drive apoptosis in a caspase-dependent manner and one explanation for the lack of apoptosis may be the disruption of this signalling pathway (215). It is however unlikely that the signalling pathway would be disrupted in two independent models where upregulation was not associated with apoptosis. The increased expression of IFI27L2B was also not explained by an increase in mitochondria biogenesis, as *Nrf1* and *CoxIV*, other mitochondrial genes, were not similarly overexpressed. The role for IFI27L2b expression is yet undetermined and requires further investigation.

In conclusion, the findings in this chapter outline a role for *Arsg, Gpld1* and *Ifi27l2b* in not only NASH but also in ASH and fibrosis injury. While this study has investigated some key characteristics associated with the changes in gene expression and the development of injury, the exact role of these genes in disease development, progression and/or amelioration is
lacking. Therefore, further studies are required to determine the cause of differential gene expression seen in this study. A large number of factors such as altered lipid metabolism, development of insulin resistance, oxidative stress, disrupted bile metabolism and inflammation are associated with the development of injury in the models investigated. One or more of these factors could act as regulators of gene expression. Additionally, some of these factors are systemically regulated and could be affected by other organs such as development of muscle insulin resistance, adipolysis and adipokine dysregulation. To specifically understand the role of these genes it is important to investigate changes in an isolated system to reduce effects from these confounding factors hence the following chapter has developed and characterised a model of fat and iron loading in normal hepatocytes to enable further investigation of these genes.
Chapter 5  Development of an In vitro Model to Investigate Iron Loading in NAFLD
5.1 Introduction

NAFLD represents a wide spectrum of diseases ranging from simple steatosis to steatohepatitis. It is characterised by triglyceride accumulation and in its more severe form – steatohepatitis – it is associated with inflammation and can lead to more severe phenotypes of end-stage liver disease and cancer (256-258). The mutation of the HFE gene leading to hereditary haemochromatosis has been one factor which has been implicated in NAFLD disease progression (6, 119, 121) and iron overload has been associated with oxidative stress and altered lipid and glucose metabolism (259). Previous studies from our laboratory have shown increased disease severity – steatohepatitis and necroinflammation – in Hfe knockout mice fed a high calorie diet. In this model, the observed pathology was associated with impaired lipid handling. It was speculated that a functional HFE may have a protective role against lipotoxicity (10).

A follow-up study, as described in chapter 3 of this thesis, explored the milieu of transcriptomic alterations associated with this animal model of Hfe-associated steatohepatitis and identified those genes with a potential role in the evolution of steatohepatitis. Further investigation of these genes in Chapter 4 identified differential expression of Arsg, Gpld1 and Ifi27l2b in the development of ASH and fibrosis. The underlying mechanisms affecting these changes could not be identified given the myriad of biochemical changes in the models investigated. In the present study, a model of fat and iron loading in a normal mouse hepatocyte cell line (AML12) was established to enable further investigation of the potential role of these candidate genes. A well-characterised in vitro model would allow investigation of the role and biochemical effects of the differentially expressed genes as well as hepatocyte mediators of co-toxic injury of fat and iron since the confounding effects of other cell types and endocrine factors that might affect hepatocyte behaviour will be excluded. Previous studies have investigated the effect of fat loading on oxidative stress, inflammatory cytokines and apoptosis (260, 261). The investigation of the effects of simultaneous fat and iron loading in cells however, is limited to only one other study which has assessed insulin responsiveness (155). The work outlined in this chapter has assessed through expression analysis the metabolic changes in the setting of concomitant fat and iron loading of normal hepatocytes in culture.

This model of fat and iron loaded hepatocytes also allowed an investigation of hepatocyte iron homeostasis in the setting of fat accumulation. Mild to moderate iron loading is common in NAFLD patients, sometimes independent of mutations in the HFE gene (7, 129) and iron
accumulation may result from impaired iron export from hepatocytes or ineffective iron sensing causing inappropriate intestinal iron uptake and subsequent hepatocyte iron accumulation.

Hepcidin is the pivotal regulator of iron homeostasis via its interaction with ferroportin on duodenal enterocytes and macrophages and this interaction leads to internalisation and degradation of ferroportin thereby reducing iron efflux from these cells into the plasma (22). There has been recent evidence for lowered hepatic hepcidin expression in rodents with steatosis (10, 161). Hepcidin expression is regulated, in part, by iron via bone morphogenetic protein 6 (BMP6) and by inflammation via interleukin 6 (IL6) (32). This study also aimed to investigate the integrity of these hepcidin signalling pathways in lipid-laden hepatocytes to determine potential mechanisms of iron loading in the setting of hepatic steatosis.

5.2 Hypothesis
Fat loading reduces hepcidin expression via the loss of integrity of the iron sensing and inflammatory signalling pathways.

5.3 Aims
The specific aims were:

1) To determine the concentration and duration of fatty acid and iron exposure sufficient to induce fat and iron loading in AML12 cells while maintaining cell viability.
2) To use this in vitro model of fat and iron loading to examine changes in gene expression associated with the co-toxic injury.
3) To investigate the integrity of the hepcidin signalling pathways in the setting of steatosis, particularly the BMP6-hepcidin axis.
5.4 Materials and Methods

5.4.1 Cell culture techniques

AML12 cells (CRL-2254, ATCC, Manassas, VA, USA) were cultured in DMEM:F12 (1:1) (Lonza, Victoria, SA, Australia) supplemented with 10 % FCS (Lonza), 1X Insulin-Transferrin-Selenium-Sodium Pyruvate (Life Technologies, Carlsbad, CA, USA) and 40 ng/ml dexamethasone (Life technologies). Cells were maintained in a 5 % carbon dioxide (CO$_2$) incubator at 37 °C. Cells were passaged 1-2 times a week.

For most experiments (unless otherwise mentioned) cells were seeded at a density of 80,000 cells/well in a 24-well plate and 10,000 cells/well in a 96-well plate. All in vitro experiments were performed three times (3 biological replicates) and results collated. Two technical replicates were also run per experiment. In some cases where explicitly required by a commercial kit, cells were seeded in triplicate per treatment. After seeding, the cells were allowed to adhere to the culture plate overnight before the media was replaced with the treatment.

5.4.2 Free fatty acid treatment

Sodium salts of oleate and palmitate (Sigma-Aldrich, St Louis, MO, USA) were utilised for fatty acid induction in a 2:1 ratio which simulates benign chronic steatosis as has been described previously (261, 262). Stocks of sodium oleate (80 mM) and sodium palmitate (40 mM) were prepared in a 0.01M sodium hydroxide solution and allowed to dissolve for at least one hour at 70 °C. Fatty acid-free BSA (8 %) (Sigma) was prepared in 1X phosphate buffered saline (PBS) and allowed to dissolve at 37 °C which was conjugated to the free fatty acids to facilitate uptake into the cells. The free fatty acid stocks were diluted 1:9 in pre-warmed BSA to make a 12 mM free fatty acid solution with 2:1 ratio of oleate to palmitate. This preparation was incubated at 37 °C to avoid fatty acids coming out of solution. The lipids were conjugated to the fatty acid-free BSA which increases the aqueous solubility of the lipids and allows their controlled uptake by the cells. The free fatty acids solution was added to pre-warmed media to make up the required final concentrations of free fatty acids (0.5 mM, 1 mM, 2 mM and 4 mM) which were then applied to the cells. The cells were treated for 12, 24 and 36 hours.

5.4.3 Iron loading

Ferric ammonium citrate (FAC) (Sigma-Aldrich, St Louis, MO, USA) was utilised to induce iron loading in cells. The upper limit for the percentage of iron in FAC compound (18.5 %) was utilised to calculate molar concentrations of iron in FAC. The desired concentration of iron (25 μM – 500
μM) was prepared by dissolving FAC in media. The solution was filter sterilised before use and was applied to the cells for 12 hours to induce iron loading.

5.4.4 Cell viability assay

The Cell Titre-Blue® Cell Viability Assay (Promega, Madison, WI, USA) uses resazurin dye to measure the metabolic capacity of the cells as an indicator of cell viability. The viable cells retain the ability to reduce resazurin to resorufin which is a highly fluorescent dye. The cell viability reagent (20 µl) was added to wells on a 96 well plate and was incubated at 37 °C for 1 h. The fluorescence intensity was measured at 560_{Ex}/590_{Em}nm in triplicate in a plate reader (Tecan, Maennedorf, Switzerland). A no cell control was used to account for background fluorescence. The cells were treated for 12, 24 and 36 hours.

5.4.5 Oil Red-O staining and quantification

Intracellular fat accumulation was determined by Oil-Red O staining: Old-Red O is a lipophilic dye which stains neutral triglycerides and lipids. Cells treated with free fatty acids were washed twice with 1X PBS. Care was taken to not disrupt the cell monolayer and to not allow the cells to remain dry for more than 30 s between washes. The cells were then fixed with 10 % formalin solution for up to 1 h. A stock solution of Oil Red-O (0.3 %, ProSciTech, Thuringowa Central, Australia) was made up in 99 % isopropanol solution. The working solution was then prepared by mixing 3 parts of the prepared stock solution to 2 parts of deionised water which was filtered and used within 2 h of preparation. After formalin fixation, the cells were washed twice with deionised water and incubated for 2-3 minutes in 60 % isopropanol followed by 5 min incubation with oil-red O working solution. The cells were then rinsed with tap water until the water ran clear and visualised under an inverted microscope. Once images were taken the cells were left in the fume hood to dry and then treated with 200 µl of dimethyl sulfoxide (DMSO) which was used as an extraction reagent. The extracted DMSO was transferred to a 96-well plate and absorbance at 540 nm was measured to quantify lipid accumulation. The absorbance of DMSO was utilised as the blank to subtract background.

5.4.6 Triglyceride extraction

Intracellular triglycerides were quantified using the Wako L-type triglyceride-M kit (Wako Diagnostics, Richmond, VA, USA). Cells were detached from the plate using 0.25 % trypsin-EDTA (Lonza). The cell pellet was resuspended in 100 µl of 0.154 M (1.15%) potassium chloride (KCl) and mixed to homogenise followed by sonication in a sonicating water bath (WiseClean D-06H, PMI-Labortechnik, Grafstal, Switzerland) at 100 % frequency on ice
times for 30 s with a 30 s recovery period. At this stage, 10 μl of the suspension was kept aside for protein determination. To the remaining homogenate, 100 μl of chloroform: methanol (2:1) was added and shaken vigorously for 30 s. Samples were centrifuged at 9,500 g for 5 min and the bottom layer was collected. The samples were then left to dry overnight in the cold room. The dried samples were reconstituted in 10 μl isopropanol: water (1:1) with 2 % triton-X solution. The samples were mixed using a vortex and sonicated in a sonicating water bath at 100 % frequency 3 times for 30 s with a 10 s recovery period. The samples were loaded on a 96-well plate with the negative control and absorbance at 590 nm was measured and triglyceride content calculated as per manufacturer’s instructions.

5.4.7 Iron quantification
Cells treated with iron were homogenised in RIPA buffer (50 mM Tris base pH 8.0, 100 mM sodium chloride, 1 % octyl phenoxypolyethoxylethanol: NP-40 and 0.5 % sodium deoxycholate). The homogenate was centrifuged at 16,000 g at 4 °C for 20 min and the supernatant was separated. 20 μl of the supernatant was mixed with 20 μl of acid reagent (3M HCl – 10 % trichloroacetic acid) and incubated at room temperature for 5 min. The mixture was centrifuged at 3000 g for 10 min at RT. Ten μl of the supernatant was added to 190 μl of chromogen reagent (0.01% Bathophenanthrolene disulphonate sodium salt and 0.1 % thioglycollic acid in Saturated Sodium acetate). Absorbance was measured at 540 nm and concentration was determined from a standard curve.

5.4.8 BMP6 treatment
Cells were serum starved for 4 hours and then treated with recombinant human BMP6 (R and D Systems, Minneapolis, MN, USA). BMP6 (50 ng/ml) was made up in cell culture media and applied to the cells for 12 hours.

5.4.9 Western blot
Western blot analysis for L-Ferritin, IFI27L2B, GPLD1, phospho-SMAD1/5/8 and phospho-STAT3 was performed as described in Chapter 2, Section 2.9. GAPDH protein was used as the loading control to normalise protein expression.

5.4.10 Gene expression analysis
Gene expression analysis was performed by real time quantitative PCR as described in Chapter 2, Section 2.6. Gene expression of all genes was normalised to Gapdh, B2mg and Btf3.
5.4.11 Statistics

All cell culture experiments, unless otherwise specified, were performed in triplicate. In each independent experiment, all treatments were performed as two technical replicates. Relative expression data from RT-qPCR analysis and protein expression data from western blot analysis was log transformed: \[ \log_{10}(x) + 1 \], to transform the data into a normal distribution. The log transformed data was subjected to a 2-way analysis of variance (ANOVA) with ‘Day of experiment’ as the blocking factor to account for variability arising due to performance of experiments on a different day and the use of cells from a different batch. The effects of the respective treatments at \( p \leq 0.05 \) were considered significant and have been reported. In experiments where an interaction of the respective treatments was found significant, the individual effects are not reported. In this case, Holm-Sidak’s post-hoc test was performed and the differences between individual groups are represented.

All statistical analysis was performed using the IBM SPSS statistics v22 (IBM Corp, Armonk, NY, USA) and graphs were generated using GraphPad prism v6.0 (La Jolla, California, USA).
5.5 Results

5.5.1 Development of an in vitro model of free fatty acid and iron loading in AML12 cells

A normal mouse hepatocyte cell line (AML12) was utilised to examine the effects of iron and fat loading. AML12 cells were firstly selected to maintain consistency of species between *in vivo* and *in vitro* analyses and secondly because they are known to retain their differentiated features after several passages and have expression of albumin, α-fetoprotein and lactate dehydrogenase (LDH), which are very similar to primary murine hepatocytes (263). AML12 cells also had the added advantage that they are not derived from a tumour cells line like most other hepatocyte cell lines such as Hepa1-6. Oleic (C18:0, unsaturated) and palmitic (C16:0, saturated) acids are the most abundant free fatty acids in normal subjects and in patients with NAFLD (264). The cells were cultured with sodium salts of oleate and palmitate in a 2:1 ratio which is the physiological ratio of abundance of these fatty acids in NAFLD (264).

Cells were cultured with increasing concentrations (0.5, 1.0, 2.0 and 4.0 mM) of free fatty acids and cell viability assays were performed. These assays indicated decreased cell viability with increasing concentration and duration of free fatty acid exposure. The FFA treatment was found to be toxic at all concentrations after 24 and 36 hours of exposure compared to the control [2 mM NaOH (*p* ≤ 0.05)]. At a concentration of 1 and 2 mM FFA the cells maintained viability after 12 hours of FFA exposure (Fig 5.1 A). Hepatocellular steatosis was assessed by microscopic analysis after staining with a lipophilic dye Oil-Red O. A dose-dependent increase in lipid accumulation was observed (Fig 5.1 D). The findings from microscopic analysis were then confirmed by triglyceride estimation and absorption spectrometry which showed intracellular lipid accumulation with 2 mM FFA exposure for 12 hours (Fig 5.1 B and C). The triglyceride assay and Oil-Red O quantification displayed a distinct increase at 4 mM FFA concentration however at this stage there was significant cell death due to cytotoxicity.

Iron loading was performed using ferric ammonium citrate (FAC). Increasing concentrations of iron from 25 μM to 500 μM in FAC were used to determine the appropriate concentration to facilitate iron loading. A dose-dependent increase in iron levels was observed after 12 hours of treatment with a significant accumulation observed with 100, 200 and 500 μM of FAC compared to the control (*p* ≤ 0.05, Fig 5.1 E). Iron loading achieved using 100 μM iron was 588.39 μmol/10^6 cells (i.e. x 7 greater than the control). FFA administration did not have any
effect on iron loading in the cells. Thus, in this study 2 mM FFA and 100 μM FAC with 12 hour exposure was used to induce fat and iron loading without inducing cytotoxicity.

**Fig 5.1: Free fatty acid and iron accumulation in AML12 cells.** Cell were exposed to increasing concentration free fatty acid (FFA) from 0 to 4 mM A) Dose and time-dependent viability of cells was assessed by fluorometric quantitation B) Triglyceride accumulation was quantified by spectrophotometric analysis and C) Lipid accumulation was quantified by measuring the absorbance of lipophilic dye Oil-red O, 12 hours post treatment. D) Intracellular lipid accumulation evidenced by Oil-red O staining at 20X magnification. E) AML12 cells were incubated with FFA and increasing concentrations of ferric ammonium citrate (FAC) and the dose-dependent accumulation of iron in cells was determined by an iron quantitation assay. Data is represented as mean ± SEM from 3 independent experiments. The * represents significance from a 2-way ANOVA for comparison between treatment groups with the untreated control at p ≤ 0.05.
5.5.2 *Increased expression of genes involved in fatty acid oxidation in lipid loaded AML12 cells*

The expression of genes which regulate lipid metabolism was examined. In these cells, expression of sterol regulatory element-binding transcription factor 1 (*Srebf1*), the main transcription factor responsible for the regulation of *de novo* lipogenesis, was downregulated by FFA treatment alone (*p* = 0.026). This reduction in expression was not seen with iron treatment alone or the combination of FFA and iron, suggesting that iron abrogates the downregulation of *Srebf1* by FFA treatment. Acetyl-coA carboxylase 1 (*Acc1*) and fatty acid synthase (*Fasn*) are the downstream target genes of *Srebf1* and are involved in fatty acid synthesis. The expression of the genes for both these proteins was altered in a pattern similar to that of *Srebf1* (Fig 5.2 A-C).

Peroxisome proliferator receptor alpha (*Ppar-α*) is the crucial regulator of fatty acid oxidation and the administration of iron appeared to significantly effect gene expression (*p* = 0.047, Fig 5.2 D). Carnitine palmitoyltransferase 1A (*Cpt1a*), is the rate-limiting enzyme responsible for the transport of fatty acids into the mitochondrial inner membrane and expression of this gene was increased two fold with FFA treatment (*p* ≤ 0.001, 5.2 E), and the combination of FFA and iron administration also significantly increased expression by 3-fold (*p* ≤ 0.001) compared to the control. Additionally, the combination of FFA and iron had significantly higher expression in comparison to the FFA treatment alone (*p* = 0.017) suggestive of an increase in fatty acid β-oxidation in cells treated with the co-administration of FFA and iron. Peroxisome proliferator receptor gamma (*Ppar-γ*) regulates fatty acid storage and the administration of iron appeared to abrogate the upregulation observed with FFA alone (*p* ≤ 0.01, Fig 5.2 F). The overall gene expression changes indicate an environment of reduced lipogenesis and increased fatty acid β-oxidation in AML12 cells treated with FFA.

5.5.3 *FFA and iron co-administration increases L-ferritin gene expression in AML12 cells*

The expression of the genes which transcribe proteins involved in iron metabolism were investigated to assess the changes in expression with iron loading and the co-toxicity of FFA and iron loading. Iron loading alone did not appear to alter *Fpn* expression. The combination of FFA and iron treatment however significantly increased *Fpn* gene expression (*Fpn*: *p* ≤ 0.01, Fig 5.3 A).
Fig 5.2: **Relative expression of de novo lipogenesis and β-oxidation genes.** AML12 cells were treated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h. mRNA expression of A) Srebf1, B) Acc1, C) Fasn, D) Ppar-α, E) Cpt1a and F) Ppar-γ was quantified by RT-qPCR. The data is represented as mean ± SEM from 6 independent experiments. Significant effects are reported from 2-way ANOVA. The bars represent significance from a Holm-Sidak’s post-hoc test at *p ≤ 0.05. Sterol regulatory element-binding transcription factor 1 (Srebf1), acetyl-CoA carboxylase (Acc1), fatty acid synthase (Fasn), peroxisome proliferator-activated receptor alpha (Ppar-α), carnitine palmitoyltransferase 1a (Cpt1a), peroxisome proliferator-activated receptor gamma (Ppar-γ).
Administration of iron alone surprisingly resulted in the reduction of L-ferritin while the combination of FFA and iron treatment increased (p ≤ 0.001) its expression. Transferrin receptor 1 (Tfr1) expression was increased with iron treatment (p ≤ 0.01) alone and was reduced with co-administration of FFA and iron (p = 0.01). Iron administration appeared to have a reverse effect to that expected on expression of L-ferritin and Tfr1. While this was initially surprising, previously published data has evidenced a similar effect on iron metabolism genes despite iron loading of cells (33, 265). The iron loading phenotype of the cells was confirmed by quantification of L-ferritin protein which increased with iron treatment and a further increase with the combination of FFA and iron treatment was observed (p ≤ 0.001) (Fig 5.3 B).

Fig 5.3: Relative expression of genes which transcribe proteins of iron metabolism. AML12 cells were treated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h. A) Relative mRNA expression of Fpn, L-ferritin and Tfr1. B) L-Ferritin representative western blot image and protein expression analysis. The significant effects are reported from 2-way ANOVA. Data is represented as mean ± SEM from 3-6 independent experiments. The bars represent significance from Holm-Sidak’s post-hoc test at *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. Ferroportin (Fpn), transferrin receptor 1 (Tfr1).
5.5.4 Cellular stress response to FFA and iron treatment

The expression of an inflammatory cytokine chemokine (C-C motif) ligand 5, *Ccl5*, transforming growth factor (*Tgf*-β), mitochondrial superoxide dismutase 2 (*Sod2*) (a surrogate marker of oxidative stress), and the pro-apoptotic gene Bcl2-associated X protein (*Bax*) were investigated as these are important components of pathways of liver injury. *Ccl5* expression was reduced with iron treatment alone but the co-administration of FFA and iron increased gene expression (*p* ≤ 0.01, Fig 5.4 A). *Tgf*-β expression was unaltered with FFA treatment alone but treatment with iron and the combination of FFA and iron reduced gene expression (*p* ≤ 0.01, Fig 5.4 B). The mRNA expression of *Sod2* was increased (*p* ≤ 0.01, Fig 5.4 C) with FFA administration and gene expression of pro-apoptotic gene *Bax* was reduced with FFA treatment (*p* = 0.039, Fig 5.4 D). Together this data points toward increased inflammatory and proliferative stimulus in cells with FFA and iron induced co-toxicity.

**Fig 5.4:** Relative expression of markers of apoptosis, oxidative stress and inflammation. AML12 cells were treated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h. Real-time PCR was used to determine mRNA expression of A) *Ccl5*, B) *Tgf*-β, C) *Sod2* and D) *Bax*. The significant effects are reported from 2-way ANOVA. Data is represented as mean ± SEM from 6 independent experiments. Chemokine (C-C motif) ligand 5 (*Ccl5*), transcription growth factor β (*Tgf*-β), mitochondrial superoxide dismutase 2 (*Sod2*), bcl2-associated X protein (*Bax*).
5.5.5 Expression of candidate genes identified by transcriptomic analysis

Previous chapters in this thesis have investigated the expression of \textit{Arsg}, \textit{Gpld1} and \textit{Ifi27l2b} and found that while these genes were altered in part in an environment of altered lipid metabolism differential expression was also observed in fibrotic livers devoid of lipid accumulation. This study has investigated expression analysis of these genes in an isolated setting to assess the particular effect of FFA and Fe loading in hepatocytes.

Firstly, a time course analysis of was performed to assess if the genes had a differential pattern of gene expression with prolonged exposure to FFA and the accumulation of lipid droplets. Gene expression was analysed over 12 h after which cells displayed cytotoxicity. The expression of \textit{Arsg} and \textit{Gpld1} was altered in a time and FFA dependent fashion with an increase in expression at early time points (4 h post-treatment, \( p \leq 0.01 \), Fig 5.5 A and B) and a decline at 10 and 12 h of FFA exposure. Basal expression of these genes (without FFA treatment) appeared to increase with time in culture but this change was not significant. There was no change in \textit{Ifi27l2b} expression with time in culture (Fig 5.5 C).

\textit{Arsg} mRNA levels were significantly reduced with FFA treatment alone \((p = 0.013\), Fig 5.6 A). This reduction in expression with FFA treatment is in contrast to the expression seen in response to a HCD \textit{in vivo}. Similarly \textit{Ifi27l2b} expression, which was strikingly increased \textit{in vivo} with HCD feeding and in fibrotic livers, was reduced with FFA and the combination of FFA and iron in comparison with the control. These effects of FFA and Fe on gene expression were however not significant (FFA effect \( p = 0.061 \), Fig 5.6 A). \textit{Gpld1} expression appeared to reduce with FFA and iron treatment, similar to the \textit{in vivo} observation, but overall changes were not significant. Protein expression of GPLD1 on the other hand was increased with iron treatment in comparison to the control (Fig 5.6 B).

5.5.6 Hepcidin signalling pathways in FFA and iron loaded hepatocytes

AML12 cells treated with FFA, iron and the combination of FFA and iron were supplemented with BMP6 to investigate the integrity of the BMP-SMAD regulatory pathway under these conditions. As expected, those cells treated with iron alone or iron and FFA displayed an increase in iron concentration and ferritin protein (Fig 5.7 A) which was not altered by the administration of BMP6. The expression of genes downstream of BMP6 – \textit{Hamp1}, \textit{Id1}, \textit{Smad7} and \textit{Atoh8} was increased \((p \leq 0.05)\) in AML12 cells treated with BMP6 (50 ng/ml) alone, indicating an intact BMP6 signalling pathway (Fig 5.7 B).
Fig 5.5: Time course expression analysis of Arsg, Gpld1 and Ifi27l2b after 2mM free fatty acid administration. AML12 cells were treated with 2 mM FFA over a duration of 12 h and mRNA expression of A) Arsg, B) Gpld1 and C) Ifi27l2b was quantified by RT-qPCR. The data is represented as mean ± SEM from 3 independent experiments. Analysis from a 2-way ANOVA is reported where *p ≤ 0.05 for comparison of FFA treated cells to the cells treated with FFA for 15 m and #p ≤ 0.05 for FFA treated cells to the cells treated with FFA for 4 h. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b).

There was a marked attenuation in the expression of hepcidin in response to BMP6 stimulation in cells treated with FFA alone as well as cells treated with iron and FFA compared to AML12 cells treated with BMP6 alone (Fig 5.7 B). Other genes downstream of BMP6 – Id1, Smad7 and Atoh8, also displayed a reduction in expression with FFA and iron treatment (p ≤ 0.01). Phosphorylated SMAD1/5/8 protein, an upstream transcription factor, was assessed and a blunted response to BMP6 stimulation was similarly observed in each group of cells (p ≤ 0.01, Fig 5.8).
Stimulation with IL6, also a hepcidin inducer, did not show significant induction of hepcidin (Fig 5.9 A) expression and there were no differences observed with FFA and iron treatment either. Protein expression of phosphorylated STAT3, an IL6 signalling intermediary, with IL6 treatment was reduced in response to FFA and iron administration ($p \leq 0.01$, Fig 5.9 B).

![Graphs showing relative expression of Arsg, Gpld1, and Ifi27l2b](image)

**Fig 5.6:** Expression analysis of candidate genes identified by transcriptomics analysis. A) Real-time PCR was used to determine mRNA expression of Arsg, Gpld1 and Ifi27l2b after treatment with 2 mM FFA, 100 μM iron and the combination FFA + Fe for 12 h B) Protein quantification with representative western blot image of GPLD1 and IFI27L2B, expression was normalised to expression of GAPDH. The data is represented as mean ± SEM from 3-6 independent experiments. Significant effects are reported from 2-way ANOVA. The bars represent significance from Holm-Sidak’s post-hoc test at $^*p \leq 0.05$. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b).
**Fig 5.7: Blunted BMP6 signalling with FFA and iron treatment.** AML12 cells were treated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h and were also supplemented with BMP6 (50 ng/ml) for 12 h. A) Iron quantification, Ferritin protein quantification with representative western blot image. B) Gene expression of *Hamp1*, *Smad7*, *Id1* and *Atoh8* was analysed by RT-qPCR. The data is represented as mean ± SEM from 3 independent experiments. Analysis from a 2-way ANOVA is reported where #p ≤ 0.05 for comparison of BMP6 stimulated cells to SFM controls and *p ≤ 0.05 for comparison of cells treated with FFA/iron and FFA + iron and stimulated with BMP6 to cells stimulated with BMP6 alone from Holm-Sidak’s post-hoc tests. Bone morphogenetic protein 6 (BMP6), serum free media (SFM), hepcidin (*Hamp1*), mothers against decapentaplegic homolog 7 (*Smad7*), DNA binding protein inhibitor 1 (*Id1*) and atonal homolog 8 (*Atoh8*).
Fig 5.8: Blunted SMAD1/5/8 phosphorylation with FFA and iron (Fe) treatment. SMAD1/5/8 (phosphorylated) representative western blot image and protein quantification. The data is represented as mean ± SEM from 3 independent experiments. Analysis from a 2-way ANOVA is reported where #p ≤ 0.05 for comparison of BMP6 stimulated cells to SFM controls and *p ≤ 0.05 for comparison of cells treated with FFA/iron and FFA + iron and stimulated with BMP6 to cells stimulated with BMP6 alone from Holm-Sidak’s post-hoc tests. Serum free media (SFM).

Fig 5.9: No effect of IL6 stimulation on hepcidin expression. AML12 cells were treated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h and were also supplemented with IL6 (50 ng/ml) for 12 h. A) Relative mRNA expression of Hamp1 from RT-qPCR analysis B) STAT3 (phosphorylated) protein quantification with a representative western blot image. Signal transducer and activator of transcription (STAT3). The data is represented as mean ± SEM from 2 independent experiments. Analysis from a 2-way ANOVA is reported where #p ≤ 0.05 for comparison of IL6 stimulated cells to SFM controls and *p ≤ 0.05 for comparison of cells treated with FFA/iron and FFA + iron and stimulated with IL6 to cells stimulated with IL-6 alone from Holm-Sidak’s post-hoc tests. Interleukin 6 (IL6), hepcidin (Hamp1), serum free media (SFM).
5.6 Discussion

Iron overload associated with the HFE gene mutations has been linked with the progression of simple steatosis to steatohepatitis and end-stage liver disease (6-8, 266). In this study an *in vitro* hepatocyte model of steatosis with concomitant iron loading was developed. This model was used to investigate potential metabolic mechanisms of concomitant fat and iron loading as well as study the integrity of hepcidin regulation.

The results demonstrate that iron loading does have an effect on lipid metabolism in AML12 hepatocytes. Iron loading appeared to abrogate the reduction of *de novo lipogenesis* gene expression (*Srebfl, Acc1* and *Fasn*) observed with FFA treatment alone. This possibly indicates increased *de novo* lipogenesis capacity of cells exposed to FFA and iron. In the presence of FFA, co-administration of iron also resulted in increased *Cpt1a* expression facilitating mitochondrial uptake of FFA and potentially increased mitochondrial β-oxidation. Additionally, iron treatment normalised the expression of *PPAR-γ*, which regulates fatty acid storage. The overall effect of iron administration on gene expression is suggestive of an increase of the FFA pool, increased mitochondrial β-oxidation and reduced storage of FFA into lipid droplets. The development of lipid droplets is central to the development of hepatic steatosis and in a sense is a protective mechanism to reduce the pathogenesis of lipotoxicity (267). It can also be argued that an increase in *de novo* lipogenesis contributes to an existing pool of FFA and the increase of mitochondrial β-oxidation contributes to oxidative stress (268) in this model, all of which indicate a role of iron in exacerbating FFA induced injury. This hypothesis is consistent with the observed increased expression of pro-inflammatory cytokine *Ccl5* also indicative of a more severe phenotype for simultaneous FFA and iron loading.

Three genes of interest in relation to the progression of NAFLD were identified in a previous transcriptome analysis. In this study, one of those genes – *Gpld1* – had a similar gene expression in a FFA rich environment as was found *in vivo*. The expression of this gene was also found to be bi-phasic, and was increased at early stages of FFA exposure and declined at the end of treatment. Previous results in this thesis reported a reduction of hepatic and serum GPLD1 in mice which developed steatohepatitis and these results were contrary to previously reported patient studies which have observed an increase in hepatic and serum GPLD1 (234). The bi-phasic expression of *Gpld1* observed in this thesis addresses this opposing result and identifies GPLD1 as a potential biomarker to predict the development of steatohepatitis. In contrast to *Gpld1, Arsg* and *Ifi27l2b* had different gene expression changes to that seen *in vivo* and it is
likely that these genes might be responsive to other stimuli associated with NAFLD progression such as oxidative radicals and inflammatory response, and thus not have a primary pathogenic role in this condition. While these changes in expression observed in response to FFA and iron loading are not all identical to that observed in vivo, they represent novel genes with a yet unrecognised role in the development of liver injury and will be investigated further in this thesis to attempt to delineate the underlying mechanisms.

In this study, an attenuated hepcidin response to BMP6 in cells treated with FFA was identified. Reductions in hepatic hepcidin expression and serum hepcidin have been found in NAFLD (10, 161, 266) and to my knowledge this is the first study to demonstrate a mechanism for the reduced hepcidin expression in a FFA-rich environment. The BMP6 signalling cascade was intact in serum-starved AML12 cells. However, the expression of many of the target genes of BMP6 was blunted in response to its administration in the presence of FFA treatment. The reduced expression of hepcidin in response to BMP6 in FFA treated cells is explained by the reduced activation of SMAD1/5/8 - the phosphorylation of which is essential for BMP6 signalling. Interestingly, the stimulation of cells with IL6, an inflammatory cytokine which induces hepcidin expression (30, 269, 270), did not significantly increase hepcidin expression. STAT3 phosphorylation, an IL-6 signalling intermediary was induced with IL6 administration but this activation did not translate into increased hepcidin expression. Like other studies, results from this chapter have reported that BMP6 is a more potent inducer of hepcidin than IL6. While FFA altered the activation of both signalling pathways, it had a more significant effect on the transcription of BMP6 target genes.

In summary, this study has developed a model of FFA and iron loading and displayed changes in expression which indicate a role for concomitant iron and fat loading in altering expression of genes involved in lipid metabolism. Additionally, several studies suggest that iron homeostasis is disturbed in NAFLD and findings from this study have shown an alteration of BMP6-stimulated hepcidin signalling via reduced activation of SMAD1/5/8. The exact mechanisms by which the phosphorylation of SMAD1/5/8 and BMP6 signalling are downregulated are unknown and require further investigation. Examination of the factors involved in activation of SMAD1/5/8 might indicate a potential new mechanism for increased iron loading observed in a proportion of the NAFLD cohort.
6.1 Introduction

The main aim of this thesis was to identify and investigate genes with a previously unrecognised role in the progression of Hfe-associated NAFLD. The project so far has utilised a transcriptomics approach to identify novel genes and has focussed on three of these genes, Arsg, Gpld1 and Ifi27l2b to ascertain their role in the development and progression of liver disease. Gene expression profiles have been examined in normal and steatotic livers, livers with steatohepatitis (alcoholic and non-alcoholic) and a fibrosis model in Chapter 4 of this thesis. Subsequently, the expression profiles were also studied in an in vitro model of FFA and iron loading. The gene expression of the candidate genes appeared to be modulated not only in steatotic livers but also in other liver diseases with very different pathophysiology. Consistent with this data, in vitro analysis observed downregulation of expression of Arsg and Ifi27l2b which is opposite to the observed upregulation in steatotic livers, and further confirms that FFA uptake and lipid accumulation are not the primary regulators of gene expression.

Gpld1 was identified as a gene which may be modulated by FFA intake. This observation is consistent with previous studies (234) which found an association of Gpld1 with high-density lipoproteins (HDL) and has increased in patients with NAFLD. On the other hand, no studies have described a role for Arsg and Ifi27l2b expression in liver injury or lipid metabolism defects and hence a paucity of information exists with regard to the effect of altered gene expression in a fat and iron environment. Identification of their mechanism of action will highlight potential new players in the progression of liver injury. Therefore the main aim of this study was to modulate by knockdown and overexpression, expression of candidate genes to investigate downstream effects primarily on lipid accumulation and metabolism. The overall data accumulated from the analyses so far are descriptive and do not identify gene alterations as cause or effect of injury and by use of gene silencing and overexpression studies the work described in this chapter aimed to investigate the potential roles of these genes in a fat and iron loaded environment.

Additionally, insulin resistance and inflammation (97) are essential components associated with the development of steatohepatitis and have been investigated in this study to determine their effect on candidate gene expression. Kupffer cells, the resident macrophages, also play a vital role in development of injury and chronic liver injury is associated with activation of macrophages and mounting of an inflammatory response which then promotes further
hepatocellular injury (271, 272) therefore this study has also investigated the hepatocyte and macrophage cross-talk and its subsequent effect on gene expression.

6.2 Hypotheses

Alteration of expression of candidate genes through knockdown and overexpression will significantly alter lipid accumulation in an in vitro model of fat and iron loading.

In addition it was hypothesised that insulin and inflammation, essential factors in the development of steatohepatitis, will result in changes in expression of candidate genes.

6.3 Aims

The specific aims were:

1) To knockdown and overexpress candidate genes in AML12 hepatocytes to assess downstream effects on lipid loading, insulin sensitivity and inflammatory response of fat and iron loaded hepatocytes.

2) To investigate the effect of insulin stimulation and inflammation on Arsg, Gpld1 and Ifi27l2b expression.

3) To explore a role for macrophage and hepatocyte cross-talk in inducing an inflammatory response and changes in expression of Arsg, Gpld1 and Ifi27l2b.
6.4 Materials and methods

6.4.1 Cell culture techniques
AML12 cells (CRL-2254, ATCC, Manassas, VA, USA) were as described previously in Chapter 5, Section 5.4.1. RAW264.7 cells (TIB71, ATCC) were cultured in the DMEM/F12 supplemented with 10 % FCS (Lonza) and Hepa1-6 cells (CRL-1830, ATCC) and HEK293 cells (CRL-1573, ATCC) were cultured in DMEM (Lonza) supplemented with 10 % FCS (Lonza). Cells were maintained in a 5 % carbon dioxide (CO\textsubscript{2}) incubator at 37 °C. Cells were passaged 1-2 times a week.

6.4.2 siRNA transfection
siRNA SMART pools (G.E Dharmacon, Millenium Science, Mulgrave, Victoria, Australia) were utilised to knockdown Arsg, Gpld1 and Ifi27l2b. siRNA was re-suspended as per instructions from the manufacturer. Further to this cell seeding density, concentration of siRNA and concentration of transfection reagent used was optimised for use with the AML12 cell line. All siRNA knockdown experiments, after optimisation, were performed in 24-well plates with a cell seeding density of 0.8 x 10\textsuperscript{5} cells per well. Cells were seeded 24 h prior to siRNA treatment in antibiotic free media. The siRNA was initially diluted in 1X siRNA buffer to a concentration of 5 μM. The diluted siRNA and transfection reagent were respectively mixed with serum-free media and then gently combined in a 1:1 ratio. This mixture was incubated for 20 min at room temperature following which the mixture was diluted with antibiotic free media to make up the required final siRNA concentration. Culture media from the seeded wells was taken out and replaced gently with the prepared siRNA mixture. The cells were inspected at 24 h from treatment. RNA was extracted from cells at 48 h and protein at 72 h from transfection.

6.4.3 Overexpression plasmids
The overexpression plasmid pCMV6-Kan/Neo was purchased from Origene (Rockville, MD, USA) (Fig 6.1 A). The plasmid encoded the full length cDNA clone of Arsg (1.5 kB) and Gpld1 (2.5 kB) respectively. The pcDNA3.1/zeo(+) vector encoding Ifi27l2b (900 bp) (see Appendix 4, Figure 1) was a gift from Dr Liao (Institute of Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan). The adeno-associated viral vector was a gift from Dr Jason Steel (School of Medicine, The University of Queensland) (see Appendix 4, Figure 2). The plasmids were verified by performing restriction digests and fragment size analysis against a 1 Kb+ ladder (Life Technologies) after agarose gel electrophoresis. Once the plasmids were verified, chemically competent bacteria were transformed with the plasmids and were grown
in a 50 ml sterile antibiotic-selective Luria broth. The bacterial culture was harvested and plasmid was purified (Qiagen Midi prep kit, described in section 6.4.7) for subsequent mammalian transfection experiments.

An HA-tag expressing plasmid pcDNA5FRT/TO HA-IMPDH-γ was a gift from Prof Jon Whitehead (Mater Medical Research Institute, The University of Queensland).

6.4.4 **Agarose gel electrophoresis**

The required amount of molecular biology grade agarose (Bioline, London, UK) was measured, mixed with 1X TAE buffer and microwaved to dissolve. The mixture was allowed to cool, SyBr safe DNA stain (1:10,000) (Life Technologies, Carlsbad, CA, USA) was added and poured into the casting tray. The gel was allowed to set with a comb in place. Once set, the gel was submerged in 1X TAE buffer and samples were loaded. Loading buffer (1X final concentration, New England Biolabs (NEB, Ipswich, MA, USA) was mixed with the samples and the appropriate volume was loaded into the wells alongside a 1kb+ DNA ladder (Life technologies). The agarose gel was run at 100 V and visualised on the 4000MM pro Image Station (Carestream Health, Inc, Rochester, NY, USA).

6.4.5 **Bacterial transformation**

Alpha-select Gold competent cells (Bioline) were thawed on ice and 25 μl of the bacteria was transferred to a cold tube. The bacteria were mixed gently with 2.5 μl of re-constituted plasmid and left to incubate on ice for 15 min. The bacteria and plasmid mixture was subjected to a heat shock at 42 °C for 45 s followed by immediate cooling on ice for 2 min. To the cooled mixture 240 μl of Luria broth (LB) was added and the suspension was allowed to incubate at 37 °C and 250 RPM for 1 h to activate the antibiotic resistance gene. The transformed bacteria were plated on agar plates with Kanamycin (25 μg/ml) or Ampicillin (100 μg/ml) to select for positive transformants. A transformation without any plasmid was also performed as a negative control alongside all transformations.

6.4.6 **DNA purification from agarose gel**

DNA from agarose gels was purified using the Isolate II PCR and Gel Kit (Bioline). Briefly, DNA fragment was excised from the agarose gel using a clean scalpel. The gel was incubated at 50 °C with 200 μl binding buffer for 10 min. When completely dissolved the sample was loaded onto a column and centrifuged for 30 s at 11,000 g. The flow through was discarded and column was washed twice with 700 μl of wash buffer and centrifuged for 30 s at 11,000 g.
The column was centrifuged again at 11,000 g for 1 min to remove all residual ethanol from the wash step. Finally, 15 μl elution buffer was added to the centre of the column, incubated at RT for 1 min and centrifuged at 11,000 g for 1 min. The purified DNA was collected in a fresh tube and stored at 4 °C until used.

6.4.7 Plasmid purification (mini prep)
Plasmid mini preps were prepared using Isolate II Plasmid mini kit (Bioline). 1 ml of overnight grown bacterial culture was centrifuged at 11,000 g for 1 min to pellet the bacteria. The cells were re-suspended in 250 μl resuspension buffer P1 and then mixed with 250 μl lysis buffer P2 to lyse the bacteria. The suspension was mixed by inverting a few times and incubated at RT until the lysate appeared clear. The lysate was cleared by adding 300 μl neutralisation buffer and centrifuged for 5 min at 11,000 g. The cleared supernatant was transferred to a column and centrifuged at 11,000 g for 1 min to allow the DNA to bind to the column. The column was washed with wash buffer PW1 and centrifuged at 11,000 g for 1 min. The column was centrifuged again at 11,000 g for 2 min to remove all residual ethanol from the wash step. Finally, 30 μl elution buffer P was added to the centre of the column, incubated at RT for 1 min and centrifuged at 11,000 g for 1 min. The purified plasmid was collected in a fresh tube and stored at 4 °C until used.

6.4.8 Plasmid purification (midi prep)
Plasmid midi preps were prepared using QIAfilter Plasmid Midi Kit (Qiagen, Hilden, Germany). 50 ml of overnight grown bacterial culture was centrifuged for 15 min at 6000 g at 4 °C. The pellet was re-suspended in 4 ml buffer P1 and then mixed with 4 ml of buffer P2. This mixture was allowed to incubate for 5 min at RT. To lyse the bacteria, 4 ml of buffer P3 was added to the mixture and mixed by inverting until the mixture turned colourless. The mixture was transferred to the QIAfilter cartridge and allowed to incubate for 10 min at RT. A QIAtip was equilibrated by allowing 4 ml of buffer QBT to pass through the column twice by gravity flow. The nozzle of the QIAfilter cartridge was placed in the QIAtip and the lysate from the cartridge was transferred onto the column by pushing down a plunger. The lysate was allowed to enter the column by gravity flow and was subsequently washed twice with 10 ml of buffer QC. The DNA was eluted with 5 ml of pre-warmed (65 °C) buffer QF. Plasmid DNA was precipitated by adding 3.5 ml isopropanol and then centrifuged for 60 min at 5000 g at 4 °C. The supernatant was carefully discarded and the pellet was washed with 70 % EtOH. Another centrifugation for 60 min at 5000 g at 4 °C was carried out. The supernatant
was discarded and the pellet was allowed to air-dry. Once dry, the pellet was dissolved in 1X TE buffer, pH 8.0. The DNA was quantified by measuring the absorbance at 260 nm using the Tecan spectrophotometer (Tecan, Männedorf, Switzerland) and purity was assessed by determining the 260/280 nm absorbance ratio. Plasmid DNA was stored at 4 °C until used.

6.4.9 Plasmid transfection
All overexpression experiments were performed in 24-well plates with a cell seeding density of 0.8 x 10⁵ cells per well. Cells were seeded 24 h prior to transfection in antibiotic free media. Lipofectamine 3000 reagent (Invitrogen, Life technologies) was used to perform cellular transfections. The transfection reagent was used at the concentration of 1.5 μl per well and prepared by mixing with serum-free media. The overexpression plasmid (500 ng/well) was mixed with serum free media and P3000 reagent (2 μL/μg DNA). The transfection reagent and the DNA were then gently mixed in a 1:1 ratio and left to incubate for 5 min at RT. The DNA-lipid complex was then added to the cells. The cells were monitored and RNA was extracted from cells at 48 h and protein at 72 h from transfection.

6.4.10 Restriction enzyme digest
High fidelity restriction enzymes (New England Biolabs, Ipswich, MA, USA) were utilised with 10X NEB buffer. One micro litre (10 units) of enzyme was used to digest 1 μg of plasmid DNA with 5 μl NEB buffer in a total 50 μl reaction volume. The digest was performed for 1 h at 37 °C. Where required, heat inactivation was performed at 65 °C for 20 min.

6.4.11 Cloning
Cloning primers were designed to insert specific restriction endonuclease sites before and after the gene of interest, and to insert an HA-tag at the C-terminus of the gene. A traditional cloning method was followed to insert the respective sequences into the adeno-associated viral vector and has been described in detail later in this chapter (Chapter 6, Section 6.5.2.1).

6.4.12 Free fatty acid treatment
FFA treatment of AML12 hepatocytes was performed as per the protocol described previously in Chapter 5, Section 5.4.2.
6.4.13 Iron treatment
Iron treatment with ferric ammonium citrate of RAW264.7 macrophages (kindly gifted by Dr Antje Blumenthal, Diamantina Institute, Brisbane, Australia) and AML12 hepatocytes was performed as per the protocol described previously in Chapter 5, Section 5.4.9.

6.4.14 Insulin treatment
Twenty four hours after seeding, AML12 cells were treated with the vehicle, FFA, iron and the combination of FFA and iron as described previously in Chapter 5, Section 5.4.2 and Section 5.4.9. The treatment however was made up in AML12 media without Insulin-Transferrin-Selenium (ITS). At 8 h from FFA and iron treatment, media was removed and replaced with 100 nM Insulin (Sigma, St Louis, MO, USA) made up in AML12 media without ITS. After 4 h of treatment cells were harvested for RNA and protein extraction respectively.

6.4.15 Lipopolysaccharide (LPS) treatment
RAW264.7 macrophages were seeded at 0.1 X 10⁶ cells per well in a 24-well plate. After 24 h, 100 ng/ml LPS (Sigma) was made up in complete media and applied to cells. After 4 h the cells were monitored for activation and media was removed and replaced with complete media (LPS-free). The cells were incubated for another 4 h after which macrophages were collected for RNA and protein extraction respectively.

6.4.16 LPS and iron treatment of RAW264.7 macrophages and treatment of AML12 hepatocytes with conditioned media
RAW264.7 macrophages were treated with LPS, iron or left untreated as described in section 6.4.12 and 6.4.14 above. After 8 h of treatment, conditioned media from the LPS, iron and untreated macrophages was collected. The conditioned media was centrifuged at 4 °C for 2 min at 400 g to pellet any dead cells in the media. The media was allowed to warm to RT and then applied to FFA and iron or untreated AML12 cells. This protocol has been outlined in the schematic in Fig 6.1.
RAW264.7 Macrophages

AML12 Hepatocytes

Fig 6.1: Schematic for the experimental procedure for LPS and Fe treatment of RAW264.7 macrophages and subsequent conditioned media treatment of AML12 hepatocytes. RAW264 macrophages were treated with LPS (100 ng/ml) or Iron (Fe, 100μM) and simultaneously AML12 cells were treated with FFA, Fe or the combination. After 4 h of treatment with LPS, LPS-containing media was removed and replaced with fresh LPS-free media and allowed to incubate for another 4 h. After 8 h of incubation, the media from RAW264.7 cells was collected, spun down to remove dead cells and then applied to the fat and iron loaded AML12 hepatocytes. The RAW264.7 cells were harvested after the media was collected. The AML12 cells were allowed to incubate with the conditioned media for 4 h before cells were harvested for RNA extraction.

6.4.17 Gene expression analysis

Gene expression analysis was performed by real time quantitative PCR (RT-qPCR) as described in Chapter 2, section 2.6. Gene expression of all genes was normalised to the geometric mean of expression for Gapdh, B2mg and Btf3.

*Gapdh* was not utilised for normalisation of gene expression in knock-down experiments since it was used as the positive control in these experiments. Only B2mg and Btf3 gene expression were utilised for normalisation for knock down experiments.

6.4.18 Western blot

Western blot analysis for Arylsulfatase G (ARSG), Glycosylphosphatidylinositol phospholipase D1 (GPLD1), Interferon alpha-inducible protein 27 like 2b (IFI27L2B), HA-tag and phospho-AKT was performed as described in Chapter 2, section 2.9. GAPDH protein was used as the loading control to normalise protein expression.
6.4.19 Immunofluorescence

Cells grown on sterile coverslips in 24-well plates were treated as per experimental conditions. Upon completion of treatment duration, the cells in the culture plate were gently washed with phosphate Buffered Saline containing 1mM calcium chloride and 1mM magnesium chloride (PBS-CM). The cells were fixed using ice cold 3 % paraformaldehyde (PFA: 9 % PFA was dissolved in PBS-CM to make up final concentration) for 15 min at RT. The cells were rinsed with PBS-CM and permeabilised with 0.05 % saponin for 15 min at RT. The primary antibody was made up in a solution containing 5 % foetal calf serum (FCS), 5 % donkey serum and 2 % bovine serum albumin (BSA) [FDB] at the required concentration and applied to the cells. The cells were incubated in a humidified chamber for 1 h at RT and then rinsed 3 times with PBS-CM. The secondary antibody was prepared in FDB and applied to cells. The cells were incubated with secondary antibody for 1 h at RT in a humidified chamber and then rinsed 3 times in PBS-CM. The cells were then mounted on slides with mounting media containing DAPI (Santa Cruz biotechnologies, Dallas, TX, USA).

6.4.20 Cellular imaging

Light microscopy was performed using the Olympus CKX41 (Shinjuku, Tokyo, Japan) and fluorescence microscopy was performed using the Nikon eclipse Ti (Melville, NY, USA).

6.4.21 Statistical analysis

All cell culture experiments, unless otherwise specified, were performed in triplicate. In each independent experiment, all treatments were performed as two technical replicates. Relative expression data from RT-qPCR analysis and protein expression data from western blot analysis was log transformed: \( \log_{10}(x) + 1 \), to transform the data into a normal distribution. The log transformed data was subjected to a 2-way analysis of variance (ANOVA) with ‘Day of experiment’ as the blocking factor to account for variability arising due to performance of experiments on a different day and the use of cells from a different batch. The effects of the respective treatments at \( p \leq 0.05 \) were considered significant and have been reported. In experiments where an interaction of the respective treatments was found significant, the individual effects are not reported. In this case, Holm-Sidak’s post-hoc test was performed and the differences between individual groups are represented.

Where three independent groups were compared, a 1-way ANOVA was performed with ‘Day of experiment’ as the blocking factor. When an overall difference between the groups was
found significant at $p \leq 0.05$, Tukey’s post-hoc test was performed and the differences between individual groups are reported.

All statistical analysis was performed using the IBM SPSS statistics v22 (IBM Corp, Armonk, NY, USA) and graphs were generated using GraphPad prism v6.0 (La Jolla, California, USA).
6.5 Results

6.5.1 Investigating the role of candidate genes using RNA interference

After assessing changes of candidate genes in a fat and iron environment as detailed in Chapter 5, Section 5.5.5 I sought to assess molecular changes after silencing gene expression. RNA interference is a widely used technique for gene silencing and is one which was adopted to achieve gene silencing in vitro. Initial optimisation for the appropriate knockdown conditions was performed using Gapdh siRNA which was a positive control in all experiments. The various optimisations performed are outlined in Table 6.1.

Table 6.1: List of the conditions tested to optimise for efficient gene silencing.

<table>
<thead>
<tr>
<th>Optimisation performed</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of siRNA</td>
<td>10nM – 100nM</td>
</tr>
<tr>
<td>Transfection reagent</td>
<td>Dharmafect 1 – 4, Lipofectamine 3000</td>
</tr>
<tr>
<td>Concentration of transfection reagent</td>
<td>1 – 2.5 μl/well</td>
</tr>
<tr>
<td>Cell seeding densities</td>
<td>0.5 - 0.8 X 10^5 cells/well</td>
</tr>
<tr>
<td>Knockdown in alternative cell line</td>
<td>Hepa1-6 (Mus musculus tumour cell line)</td>
</tr>
</tbody>
</table>

Initially, AML12 cells were seeded at two cell densities which allowed 60-80 % cell confluence 24 h post seeding which was deemed by the manufacturer as the most appropriate concentration to perform siRNA-mediated knockdown. Transfection was performed using 10 and 25 nM of Gapdh siRNA and non-targeting (NT) siRNA with Dharmafect 1 (D1) transfection reagent. The cells were inspected at 24 h post transfection and drastic changes in cell morphology were observed. The cells had shrivelled and lost cell to cell contact (Fig 6.2 A). These changes in morphology were observed in cells treated with NT siRNA as well as Gapdh siRNA therefore the changes were unlikely a result of gene silencing but rather due to cell toxicity from the concentration and/or the formulation of siRNA or the transfection reagent utilised. Despite the observed changes in morphology, adherent cells were found to have between 50-95 % of Gapdh expression knockdown relative to the expression in the non-targeting siRNA treated controls (Fig 6.2 B).

To investigate the source of cytotoxicity, different Dharmafect formulations (D2, D3 and D4) and an intermediate concentration of siRNA (12.5 nM) compared to the previous experiment was utilised. Neither the use of the different transfection reagents nor the lowered siRNA
concentration helped with the cell toxicity. The gene silencing observed was also lower than previously observed with D1 reagent (Fig 6.3). Since cell toxicity was not an effect of the Dharmafect formulation which was utilised, all the remaining optimisations were performed using D1 reagent as it provided the most efficient gene silencing. To account for cell toxicity, the cell seeding density was increased to 0.8 X 10^5 cells/ well. This seeding density provided almost 100 % cell confluence at 24 h from seeding which was over the suggested (optimal) confluence. At this seeding density however the cells looked healthy at both 24 and 48 h post transfection.

At this seeding density, transfection was performed for Gapdh, Hfe and the 3 candidate genes Arsg, Gpld1 and Ifi27l2b. Gapdh silencing efficiency, as determined by RT-qPCR analysis, was 97 % and Hfe expression was reduced by 95 % with use of 25 nM siRNA. Surprisingly though, similar gene silencing for Arsg, Gpld1 and Ifi27l2b was not observed. Arsg and Gpld1 expression remained unchanged and Ifi27l2b expression was found to be reduced by only 20-40 % (Fig 6.4).

The lack of gene silencing could not be explained for the three genes of interest, since the positive control (Gapdh) and Hfe silencing were efficient, proving that the transfection itself was successful. This may have been an effect of insufficient siRNA to mediate knockdown hence the highest recommended concentration of the siRNA (100 nM) was utilised to overcome this problem. Consistent with previous results, Gapdh and Hfe knockdown was observed at 90 and 92 % respectively, however there were no changes in gene expression of Arsg, Gpld1 or Ifi27l2b (Fig 6.5). An alternative transfection reagent, L3000 was also trialled which did not alter the result (Fig 6.6). Lastly, a different Mus musculus tumour cell line Hepa1-6 was used with a similar result with 94 % reduction of Gapdh relative expression and no knockdown of Arsg (Fig 6.7).

In order to confirm that the primers used to detect knockdown of gene expression were specific for the correct gene, the amplification product from RT-qPCR reaction was sequenced (Australian Genome Research Facility, Brisbane, Australia). The sequencing results indicated the amplification of the correct fragment when the sequence was aligned against the Mus musculus genome. Therefore the possibility of the primers detecting the incorrect product and hence reporting a false negative result was also eliminated.

The above troubleshooting included suggestions for optimisation from Dharmacon but all troubleshooting was unsuccessful.
Fig 6.2: Cell cytotoxicity due to siRNA mediated knock-down and Gapdh silencing. A) Untreated AML12 cells had normal appearance but cells treated with siRNA appeared shrivelled. The white arrows indicate the rounded cell bodies and loss of cell to cell contact. Images taken at 20X magnification. B) Despite cell toxicity, relative Gapdh expression from cells treated with Dharmafect 1 transfection reagent and 10 nM and 25 nM siRNA had reduced gene expression. Efficiency of Gapdh knockdown was calculated as a percentage of the expression in non-targeting (NT) siRNA control. Data is presented as mean ± SEM from one experiment with two technical replicates for each condition tested.

Fig 6.3: Dharmafect (transfection) reagents D2, D3 and D4 had low silencing efficiency. AML12 cells transfected with Dharmafect reagents D2, D3 and D4 and 12.5 nM Gapdh siRNA displayed low gene silencing efficiency of GAPDH which is expressed as a percentage of expression of the non-targeting (NT) control. Data is presented as mean ± SEM from one experiment with two technical replicates for each condition tested.
Fig 6.4: Transfection with increased cell density achieved knockdown of positive controls. Transfection was performed on 0.8 x 10^5 cells/well with Dharmafect 1 reagent and 10 and 25 nM siRNA. Gapdh and Hfe gene silencing were performed as positive controls and displayed efficient knockdown of expression. Arsg, Gpld1 and Ifi27l2b did not achieve the same level of knockdown as the positive controls. Efficiency of siRNA knockdown for all genes was calculated as a percentage of the expression in the NT control. Data is presented as mean ± SEM from one experiment with two technical replicates for each condition tested. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b), non-targeting (NT).

Fig 6.5: Gene silencing optimisation of Arsg, Gpld1 and Ifi27l2b utilising the highest recommended concentration of siRNA (100 nM). Gapdh and Hfe gene silencing were performed as positive controls and displayed efficient knockdown of expression. Arsg, Gpld1 and Ifi27l2b did not achieve the same level of knockdown as the positive controls. Efficiency of siRNA knockdown for all genes was calculated as a percentage of the expression in the NT control. Data is presented as mean ± SEM from three independent experiments. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b), non-targeting (NT).
Fig 6.6: Use of Lipofectamine 3000 (L3) transfection reagent did not alter gene silencing efficiency. *Hfe* gene silencing was performed as the positive control and displays efficient knockdown of expression. *Arsg*, *Gpld1* and *Ifi27l2b* did not achieve the same level of knockdown as the positive control. Efficiency of siRNA knockdown for all genes was calculated as a percentage of the expression in the NT control. Data is presented as mean ± SEM from one experiment with two technical replicates for each condition tested. Arylsulfatase G (*Arsg*), glycosylphosphatidylinositol phospholipase D1 (*Gpld1*), interferon alpha-inducible protein 27 like 2b (*Ifi27l2b*), non-targeting (NT).

Fig 6.7: Gene silencing of *Arsg* in an alternative *Mus musculus* tumour cell line: Hepa1-6 was ineffective. siRNA transfection in Hepa1-6 was performed using Dharmafect 1 reagent and 100 nM *Arsg* siRNA, *Gapdh* (positive control) and non-target (NT) siRNA (negative control). RNA was isolated 48 h from transfection and efficiency of siRNA knockdown for was calculated as a percentage of the expression in the NT control. Data is presented as mean ± SEM from one independent experiment with two technical replicates for each condition tested. Arylsulfatase G (*Arsg*), non-targeting (NT).
6.5.2 Investigating the role of candidate genes using overexpression plasmids

As the gene silencing was unsuccessful, overexpression of the candidate genes was used to assess the effect of the candidate genes on lipid metabolism. The plasmids overexpressing the candidate genes were regulated by the widely used cytomegalovirus (CMV) promoter and were transfected into AML12 hepatocytes which resulted in a significant increase in mRNA expression \((p \leq 0.01)\) for Arsg, Gpld1 and Ifi27l2b (Fig 6.8) in comparison to control cells transfected with an empty vector. The increased mRNA expression however, did not translate to an increase in protein for any of the genes (Fig 6.8 A, B and C). Quantification of western blots did not show any differences hence the graphs have not been included. It was suspected that the antibodies for the respective transcribed proteins might not have been detecting small fold-increase in protein expression given that the endogenous protein bands were very distinct. It may also be possible that the endogenous protein expression was suppressed in the presence of plasmid overexpression such that total level of protein appeared to be unchanged.

To enable the detection of the exogenous protein expression transcribed by the overexpressing vectors, the genes were then tagged with a haemagglutinin (HA)-tag by cloning. The HA-tag was selected over other tags like green and yellow fluorescent protein, due to its small size and hence decreased likelihood to affect biochemical activities and post-translational modification of the transcribed protein.

The Ifi27l2b gene was already HA-tagged and detection of the transcribed protein with 12CA5: an antibody to the HA-peptide (a gift from Prof. Nathan Subramaniam) resulted in several non-specific bands with no overexpression observed. The antibody utilised (12CA5) was not a purified product but a supernatant from a hybridoma. Hence it might have been detecting other non-specific bands. A commercial product was purchased for latter HA-tag analysis.

6.5.2.1 Cloning

After preliminary experiments to assess the potential role of the candidate genes in fat uptake and metabolism the final aim of this study was to express these genes in vivo and assess the effect on the development of fatty liver. With this aim in mind and the documented use of adeno-associated viral vectors in targeted hepatic expression in vivo (273, 274), the adeno-associated viral (AAV) vector expressing the AAV2 inverted terminal repeats (ITRs) (Appendix 4, Figure 2) was selected as the backbone in which to clone the genes expressing the HA-tag at the C-terminus.
Fig 6.8: Transfection with overexpression plasmids results in relative mRNA overexpression but not protein. AML12 cells were either left untreated, transfected with an empty vector (pCMV/pcDNA3.1) or transfected with plasmids expressing A and D) Arsg, B and E) Gpld1 or C and F) Ifi27l2b respectively. Relative mRNA (A, B and C) and protein expression (D, E and F) was analysed 48 and 72 h after transfection. Data is represented as mean ± SEM from three independent experiments and analysed by 1-way ANOVA. *p ≤ 0.01 in comparison to untreated and empty vector controls. Arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b).

PCR primers were designed to amplify the gene of interest from the parent vector with additional restriction sites, HA-tag and stop codon on the forward and reverse primer respectively. The schematic for primer design is shown in Fig 6.9 with the primers utilised for cloning. The PCR was performed using high fidelity KOD hot start polymerase (Merck, Millipore, Darmstadt, Germany) to avoid insertion of mutations. The “Hot Start” and denaturation temperature of the PCR was kept constant for the three genes but the annealing temperature and extension time was altered with respect to the length of the genes to be amplified. Gpld1 PCR amplification was not successful initially using the KOD polymerase despite altering annealing temperature or extension times. Finally, the use of an alternative high fidelity polymerase: KAPA HiFi (KAPA biosystems, Wilmington, MA, USA) proved successful. The PCR products were purified and restriction digests were performed to verify the size of the amplified PCR inserts.

The AAV plasmid was digested with EcoRI and SalI and was also treated with alkaline phosphatase to remove phosphate from the 5’ end of the DNA to avoid re-ligation of the vector and hence reduce the probability of false positive colonies after transformation. Despite this
precaution, screening of colonies after transformation and growth on antibiotic selective agar plates grew many colonies all of which tested as negative when the colonies were screened by performing restriction digests. All the purified plasmids ran at the same size as the empty vector and colony PCR of the purified plasmid did not have any amplification curve (results not shown). Following this, various ligation and transformation conditions were tested to interrogate this problem. Ligation and transformation of the digested vector alone (no insert) developed many colonies and indicated that despite having non-complimentary sticky ends, after digestion with EcoR1 and Sal1, the vector may have been preferentially re-ligating even in the presence of the inserts and explains the large number of false positives detected.

To rectify this situation an alternative restriction enzyme, Xho1 was used. Xho1 has the same 5’ overhang sequence as Sal1 (Fig 6.10) and hence can be used interchangeably. Exploiting this feature, enabled the use of the previously amplified PCR insert with EcoR1 and Sal1 overhangs respectively rather than repeating the entire process which would entail re-designing primers, amplification, purification and verification of inserts. Instead, the vector was digested with EcoR1 and Xho1 and was ligated with the PCR inserts with EcoR1 and Sal1 overhangs (Fig 6.10). This cloning strategy was successful and screening of colonies post-transformation yielded clones positive for the insert. Sequencing of the purified plasmid confirmed that indeed the correct product was cloned and no mutations were inserted during the cloning process.

6.5.2.2 Gene overexpression using adeno-associated viral (AAV) vector

AML12 hepatocytes were transfected with the AAV plasmids coding for the respective candidate genes and significant mRNA overexpression \((p \leq 0.05)\) compared to expression in untreated and empty vector controls for all three genes Arsg, Gpld1 and Ifi27l2b was observed (Fig 6.11) by RT-qPCR analysis. Analysis for protein overexpression by western blotting using the anti-HA antibody (Santa Cruz Biotechnologies) specific for the HA-tag however did not show any bands despite long durations of exposure with the chemi-luminescence substrate. The absence of HA-tag detection could not have been due to a mutation in the coding region as the product from cloning was sequenced and verified. It was speculated that the presence of an inefficient Kozak sequence may be the reason for low or no protein transcription, it may have also been due to protein degradation after transcription or post-translational proteolytic cleavage of the C-terminus of the transcribed protein and hence degradation of the HA-tag.
Fig 6.9: Primer design schematic for cloning primers. A) Forward and reverse primer with the respective restriction endonuclease sites (EcoR1-blue and SalI-purple), HA-tag sequence (green), stop codon (red) and 18-20 nucleotides complimentary to the gene to be cloned. B) Table of primers used with the colour coding scheme depicting the different sections of the primer.

Fig 6.10: Cloning strategy used to avoid re-ligation of adeno-associated virus (AAV) vector. The PCR product of the insert for the genes of interest had EcoR1 and SalI overhangs and the AAV plasmid was digested with EcoR1 and Xho1. The restriction enzyme digest sites are depicted and show that SalI and Xho1 have the same 5’ overhang (blue box). Hence the SalI overhang would ligate with the Xho1 overhang in the presence of T4DNA ligase allowing the insert to be ligated into the AAV (adeno-associated virus) plasmid.

To investigate some of these hypotheses and considering problems encountered with transgene expression, transfection was assessed in an alternative cell line which is known to be easily transfected. Human embryonic kidney 293 (HEK 293) cell line has been extensively used as a
tool for expression analysis. This cell line was used as a positive control to verify transfection efficiency of both RNA and protein. An additional positive control of a known overexpressing plasmid with a HA-tag (HA-IMPDH) which had been previously tested (275) was used. Both HEK293 and AML12 cells were simultaneously transfected with AAV plasmids expressing Arsg, Gpld1 and Ifi27l2b respectively and HA-IMPDH. The same concentrations and reagents were utilised for the transfections across the cell lines and overexpression vectors.

Similar to previous experiments, mRNA overexpression was observed in both HEK293 and AML12 cells. The mRNA expression in HEK293 cells however was several folds higher across all the genes compared to expression in AML12 cells (Fig 6.12 A). This indicated that while overexpression was observed in AML12 cells, it was nowhere near as efficient as the HEK293 cells. Western blot analysis of HEK293 cells exhibited faint bands for transgene expression of HA-tagged-ARSG (57kDa), GPLD1 (93kDa) and IFI27L2B (30kDa) respectively (Fig 6.12 B). Contrary to this, transgene expression of HA-tagged-ARSG, GPLD1 and IFI27L2B was not observed in the AML12 cells. Additionally, the HA-IMPDH overexpressing band was very intense compared to the other proteins expressed in HEK293 cells. The AML12 cells, which seemed refractory to all other transgene expression, also expressed HA-IMPDH although the expression was lower than that observed in HEK293 cells (Fig 6.12 B).

Further assessment by anti-HA immunofluorescence in AML12 cells transfected with vectors overexpressing Arsg, Gpld1, Ifi27l2b and HA-IMPDH showed more expression of HA-IMPDH (Fig 6.12 C) compared to IFI27L2B-HA expression. This possibly represents a difference in transcription efficiency and also explains the observation of only HA-IMPDH expression on the western blot but not of ARSG, GPLD1 or IFI27L2B.

![Fig 6.11: Overexpression in AML12 cells transfected with adeno-associated viral vector.](image)

Relative gene expression compared to the control AML12 cells which were left untreated, transfected with an empty vector (AAV) or AAV/Arsg, AAV/Gpld1 or AAV/Ifi27l2b respectively. Relative mRNA expression was analysed 48 h after transfection. Data is represented as mean ± SEM from three independent experiments and analysed by 1-way ANOVA. *p ≤ 0.01 in comparison to untreated and empty vector controls. Arylsulfatase G
(Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b).

6.5.3 Reduced insulin sensitivity of hepatocytes positively correlates with Gpld1 and Ifi27l2b expression

In the absence of successful gene modulation, this project has also investigated the effect of insulin stimulation on candidate genes in fat and iron loaded AML12 cells. Insulin resistance is an important component in the development of fatty liver disease (276) and it is possible that the candidate genes found differentially expressed may be part of the insulin signalling axis and might be affected by insulin stimulation.

The stimulation of AML12 cells with insulin significantly reduced Gpld1 mRNA expression (p = 0.05) (Fig 6.13). Ifi27l2b expression as measured by RT-qPCR also appeared to have reduced in response to insulin stimulus but this effect was not significant (p = 0.186). Relative Arsg mRNA expression is consistent with previous results and had reduced with FFA treatment (p ≤ 0.01) and no effect of insulin stimulation was detected (Fig 6.13).

To ascertain if in fact the changes in gene expression were associated with reduced insulin sensitivity in fat and iron loaded hepatocytes, protein expression of phosphorylated AKT (pAKT), a protein kinase activated by insulin, in response to insulin stimulation was assessed (277). pAKT protein remained unchanged with treatment of FFA, iron and the combination of FFA and iron. With an additional stimulus of insulin (100 nM), FFA and iron loading alone did not reduce insulin sensitivity (reduced pAKT) but the combination of FFA and iron resulted in a significant reduction (p = 0.001) of the activated protein (Fig 6.14 A and D).
**Fig 6.12: Better transfection efficiency and protein expression in HEK293 compared to AML12 cells.** A) Relative mRNA expression of *Gpld1*, *Arsg* and *Ifi27l2b* in untreated cells (C), transfection with the empty vector and transfection with the gene expressing plasmid in HEK293 and AML12 cells respectively. Data is represented as mean ± SEM from one independent experiment. B) Western blot image of protein extracted from transfected HEK293 (left) and AML12 (right) cells. Bands for HA-tagged-GPLD1 (93kDa), ARSG (57kDa), IFI27L2B (30kDa) and HA-IMPDH (55kDa) proteins using the anti-HA antibody can be observed on the HEK293 (left) membrane. Only the HA-IMPDH band is visible on the AML12 (right) membrane. The loading control used is GAPDH (anti-GAPDH antibody) and it has similar band intensity across all samples on both membranes. C) Representative image of AML12 cells stained with anti-HA antibody (green) and cell nuclei stain DAPI (blue) at 10X magnification. AML12 cells transfected with HA-IMPDH (left) and with IFI27L2B-HA (right) showing a higher percentage of positive staining of HA-IMPDH compared with IFI27L2B-HA. Arylsulfatase G (*Arsg*), glycosylphosphatidylinositol phospholipase D1 (*Gpld1*), interferon alpha-inducible protein 27 like 2b (*Ifi27l2b*).

A reduction in GPLD1 and IFI27L2B protein was also observed with the combination (FFA + Fe) treatment group but statistical analysis did not demonstrate significance (Fig 6.14 B, C and D). Despite the absence of significant changes, a positive correlation between GPLD1 and pAKT was identified. This correlation was observed only in the presence of insulin stimulation ($r = 0.73$, $p \leq 0.001$, Fig 6.14 E). The correlation analysis of IFI27L2B and pAKT on the other
hand revealed a positive correlation both in the presence \( (r = 0.8, p \leq 0.001) \) and absence \( (r = 0.7, p \leq 0.01) \) of insulin stimulus (Fig 6.14 F).

**Fig 6.13:** Insulin stimulation has a significant effect on \( Gpld1 \) expression. AML12 cells were treated as indicated with 2 mM FFA, 100 \( \mu \)M iron (Fe) and the combination FFA + Fe for 8 h, followed by 4 hours of stimulation with 100 nM insulin. mRNA expression of \( Arsg \), \( Gpld1 \) and \( Ifi27l2b \) was analysed relative to expression of reference genes. Data is represented as mean ± SEM from three independent experiments. Significant effects of treatment are reported from 2-way ANOVA at \( p \leq 0.05 \). Arylsulfatase G (\( Arsg \)), glycosylphosphatidylinositol phospholipase D1 (\( Gpld1 \)), interferon alpha-inducible protein 27 like 2b (\( Ifi27l2b \)).

### 6.5.4 Inflammation drives changes of \( Arsg, Gpld1 \) and \( Ifi27l2b \) in hepatocytes and macrophages

Inflammatory changes are also key in the development of steatohepatitis (97). Therefore this study has also investigated the effect of a pro-inflammatory cytokine to assess if \( Arsg \), \( Gpld1 \) and \( Ifi27l2b \) mRNA expression may be affected by this stimulus. Treatment of AML12 cells with IL6 did not alter gene expression of \( Arsg \) and \( Gpld1 \). \( Ifi27l2b \) expression on the other hand was reduced with FFA treatment \( (p \leq 0.01) \) (Fig 6.15) and a further reduction (albeit small) was observed with IL6 treatment \( (p = 0.023) \). This result prompted investigation further of the effect of LPS-mediated inflammatory stimulation of the AML12 hepatocytes.

Chronic liver injury is associated with activation of macrophages and mounting of an inflammatory response which then promotes further hepatocellular injury. In conditions of hepatic stress, injury can occur due to cross-talk of hepatocytes and macrophages (271, 272). Therefore, the effect of conditioned media from LPS-treated macrophages, as key mediators in the development of progressive injury, on AML12 hepatocytes was also studied. Furthermore, this experiment aimed to investigate the effect of LPS treatment and iron loading on RAW264.7 macrophages with specific interest in monitoring the effect on \( Arsg \), \( Gpld1 \) and \( Ifi27l2b \).
expression. This study has utilised two methods of macrophage activation – LPS (100 ng/ml) mediated, a well-documented agent used to elicit an inflammatory response (278, 279) and treatment with iron (100 μM), since the resident liver macrophages have been identified as sites for iron loading in NASH (119).

Fig 6.14: Free fatty acids and iron co-administration reduces insulin sensitivity. AML12 cells were treated as indicated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 8 h, followed by 4 hours of stimulation with 100 nM insulin. Protein quantification of A) pAKT, B) GPLD1 and C) IFI27L2B from western blot analysis. D) Representative western blots of GPLD1, pAKT, IFI27L2B and GAPDH respectively. E) Pearson’s correlation analysis of GPLD1 and pAKT with and without Insulin stimulation. F) Pearson’s correlation analysis of IFI27L2B and pAKT with and without Insulin stimulation. Graphs are represented as mean ± SEM from 2-3 independent experiments. *p ≤ 0.001 with Insulin Vs without insulin, #p ≤ 0.05 Control vs FFA + Fe from Holm-Sidak’s post-hoc test. The correlation co-efficient is reported from Pearson’s correlation analysis. Protein kinase B (pAKT), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Iff27l2b).
IL6 expression was used as a measure of activation of an inflammatory response in both macrophages (RAW264.7) and hepatocytes (AML12). A significant increase in expression was observed in hepatocytes treated with LPS \( (p = 0.041) \) and conditioned media from activated macrophages (LPS-CM: \( p \leq 0.001 \)) compared with untreated hepatocytes (Fig 6.16 A). The increase in IL6 expression was also significantly higher with LPS-CM in comparison with LPS treated hepatocytes \( (p \leq 0.001) \). With the additional inflammatory stimulus, changes in gene expression with FFA and iron treatment were also observed but none of these alterations were found to be significant. A significant effect of LPS stimulation on gene expression of \textit{Arsg} \( (p \leq 0.001) \) and \textit{Gpld1} \( (p = 0.011) \) was observed and the reduced gene expression observed was most prominent in hepatocytes treated with LPS-CM, the cells with the most severe inflammatory phenotype (Fig 6.16 B and C). \textit{Ifi27l2b} gene expression was significantly increased with LPS stimulation \( (p = 0.037) \) with the most prominent changes with LPS-CM treatment (Fig 6.16 D).

RAW264.7 macrophages treated with iron had a spindle-like appearance and this change in morphology was more prominent with LPS treatment (Fig 6.17). While treatment with iron changed morphology of the macrophages it did not result in activation of an inflammatory response and \textit{IL6} expression remained unchanged. LPS treatment on the other hand significantly increased \textit{IL6} expression \( (p \leq 0.001) \). Consistent with this activation of IL6, a pro-inflammatory cytokine, there was a significant reduction of \textit{Arsg} \( (p \leq 0.001) \) and \textit{Gpld1} \( (p \leq 0.05) \) gene expression with LPS treatment and \textit{Ifi27l2b} expression remained unaltered. Subsequent protein analysis showed a small non-significant reduction of ARSG and a significant reduction of both GPLD1 \( (p \leq 0.01) \) and IFI27L2B \( (p \leq 0.001) \) (Fig 6.18).
Fig 6.15: IL6 treatment has an effect on expression of *Ifi27l2b*. AML12 cells were treated as indicated with 2 mM FFA, 100 μM iron (Fe) and the combination FFA + Fe for 12 h and were also supplemented with IL6 (50 ng/ml) for 12 h. Relative mRNA expression of *Arsg, Gpld1, Ifi27l2b* was analysed. Data is represented as mean ± SEM from two independent experiments. Significant effects of treatment are reported from 2-way ANOVA at $p \leq 0.05$. Arylsulfatase G (*Arsg*), glycosylphosphatidylinositol phospholipase D1 (*Gpld1*), interferon alpha-inducible protein 27 like 2b (*Ifi27l2b*).
Fig 6.16: A pro-inflammatory stimulus reduces expression of *Arsg* and *Gpld1* and increases expression of *Ifi27l2b*. AML12 cells were treated as indicated with LPS (100 ng/ml), Untreated-CM or LPS-CM. Relative mRNA expression of A) *IL6*, B) *Arsg*, C) *Gpld1* and D) *Ifi27l2b* was analysed. Data is represented as mean ± SEM from three independent experiments and significant effects of FFA/Fe treatment and LPS stimulation are reported from 2-way ANOVA. *p ≤ 0.05 when compared to untreated hepatocytes, #p ≤ 0.001 when compared to LPS treated hepatocytes. Control (C), free fatty acids (FFA), iron (Fe), lipopolysaccharide (LPS), conditioned media from untreated RAW264.7 macrophages (Untreated-CM), conditioned media from LPS treated macrophages (LPS-CM), interleukin (*IL6*), arylsulfatase G (*Arsg*), glycosylphosphatidylinositol phospholipase D1 (*Gpld1*), interferon alpha-inducible protein 27 like 2b (*Ifi27l2b*).
Fig 6.17: LPS treatment activates RAW264.7 macrophages and reduces gene expression of Arsg and Gpld1. AML12 cells were treated as indicated with iron (Fe, 100μM) or LPS (100 ng/ml) A) Representative images of untreated, iron treated and LPS stimulated RAW264 macrophages at 20X magnification. The white arrows indicate spindle-like appearance of the cells. B) Relative mRNA expression of IL6, Arsg, Gpld1, and Ifi27l2b was analysed. Data is represented as mean ± SEM from three independent experiments and analysed by 1-way ANOVA. *p ≤ 0.05, **p ≤ 0.01 in comparison to C and Fe treatment. Control (C), iron (Fe), lipopolysaccharide (LPS), interleukin (IL6), arylsulfatase G (Arsg), glycosylphosphatidylinositol phospholipase D1 (Gpld1), interferon alpha-inducible protein 27 like 2b (Ifi27l2b).
**Fig 6.18: LPS induced inflammation reduces protein expression of ARSG, GPLD1 and IFI27L2B.** AML12 cells were treated as indicated with iron (Fe, 100 μM) or LPS (100 ng/ml) and protein expression of ARSG, GPLD1 and IFI27L2B was analysed. Representative western blots are presented. Data is represented as mean ± SEM from 1-2 independent experiments and analysed by 1-way ANOVA. *p ≤ 0.01 in comparison to C and Fe treatment. Control (C), iron (Fe), lipopolysaccharide (LPS), arylsulfatase G (ARSG), glycosylphosphatidylinositol phospholipase D1 (GPLD1), interferon alpha-inducible protein 27 like 2b (IFI27L2B).
6.6 Discussion

A normal *Mus musculus* hepatocyte cell line (AML12) had been used to develop a model of FFA and iron loading to examine the effects on lipid metabolism and expression of the candidate genes found differentially expressed from transcriptomics analysis. The same cell line was also utilised to subsequently knockdown and overexpress *Arsg*, *Gpld1* and *Iffi27l2b* to analyse the downstream effects on lipid metabolism and the development of steatosis.

Despite several attempts at optimisation gene silencing was not achieved. Transfection efficiency in this cell line did not appear problematic as the positive control, GAPDH knockdown, was consistently achieved. Gene silencing for the *Hfe* gene was also performed and used as a positive control which always achieved over 90% silencing. It was not entirely clear why silencing for the candidate genes was not achieved, but it was speculated that knockdown efficiency of individual siRNA sequences may have been masked by the use of a pool of four siRNA sequences. Ordinarily, the pool of siRNA’s has been used to achieve maximum silencing efficiency with minimum off-target effects (280). It may be possible that each of the individual siRNA’s had different efficiencies of knockdown with the total effect of knockdown being masked, yielding no reduction in gene expression. In the optimisations performed, in some cases increased expression of genes was observed where a knockdown was expected and probably indicates toxic off-target effects. Other possible reasons for the lack of knock down include formation of secondary structures at the recognition site hence blocking the binding of siRNA and an increased turn-over rate of these genes and require further investigation. For future silencing experiments, which were not possible during this thesis due to the time constraints, it might be pertinent to try individual siRNA sequences or different combinations of individual siRNA sequences to achieve maximum knockdown and minimise off-target effects.

Plasmid overexpression of genes in this cell line has also proved difficult. Transfection with overexpressing plasmids yielded mRNA overexpression of the genes but these results could not be replicated at the protein level. Subsequent overexpression with a positive easily transfected cell line (HEK293) and with a previously successful overexpressing plasmid indicated that there were two problems. Firstly, the AML12 cell line had lower transfection efficiency in comparison to the HEK293 cells and secondly, the plasmids themselves did not have the same efficiency of transcription as the positive control plasmid (HA-IMPDH). All the plasmids utilised were under regulation by the CMV promoter hence this discrepancy in
transcription efficiency could not be explained by the strength of the promoter. Strength of the kozak sequence (ACCAUGG, A at -3 position from AUG - the start codon) can also affect protein translation efficiency (281). The genes of interest in this study had intact endogenous kozak sequences with the crucial A at -3 position of the start codon. The nucleotide ‘G’ at +1 position after the start codon was not altered. Hence, other alternatives of this sequence which can improve ribosomal recognition of the start codon will need to be trialled. The specific knockdown an overexpression of these genes are instrumental in determining function of these genes and will be crucial to optimise for future experiments.

Insulin resistance is associated with the development of NAFLD (276, 282) and evidence from in vitro analysis also supports a role for reduced insulin sensitivity with iron loading and the combination of FFA and iron loading (155). This study has demonstrated that FFA and iron loading alone do not have a significant effect on insulin signalling but the co-administration of fat and iron loading significantly reduces insulin sensitivity. Additionally, this study has identified a role for insulin in modulating the expression of GPLD1 and IFI27L2B. A positive correlation for GPLD1 and pAKT was found in the presence of an insulin stimulus. On the other hand IFI27L2B expression correlated with pAKT in the presence and absence of insulin stimulation. While the exact role of the changes in gene expression on insulin signalling cannot be determined from this study this finding warrants further investigation to determine if altering the GPLD1 and IFI27L2B status in a setting of fat and iron overload can improve insulin signalling and ameliorate the phenotype.

This evidence is in line with previous studies (283) where hypoinsulinaemia has increased hepatic GPLD1 expression and overexpression of Gpld1 has improved glucose tolerance (284). This is the first study to provide evidence for the role of insulin signalling of an interferon stimulated gene, Ifi27l2b. Ifi27l2b (also known as Isg12b2) belongs to the ISG12 (interferon stimulated genes 12) family of proteins that are induced by interferon α (285). This family consists of four genes (6-16, ISG12a, ISG12b and ISG12c) in humans and three genes (Isg12a, Isg12b1 and Isg12b2) in the mice (286). Isg12b1 (also known as Ifi27) expression has been reported to be upregulated by virus infection in the brain (287) and latter reports have found predominant expression in adipocytes where it inhibits adipogenic differentiation and mitochondria biogenesis (288). The findings in this study for IFI27L2B are in line with the role of ISG12b1 of the same family, and indicate an association between IFI27L2B and altered lipid status in the liver.
Stimulation of hepatocytes with IL6, a pro-inflammatory cytokine, did not alter gene expression, but treatment with conditioned media from activated macrophages, containing the milieu of the macrophage secretome resulted in reduction of Arsg and Gpld1. In keeping with its role as an interferon stimulated gene, Ifi27l2b expression was induced. The alteration in expression in response to conditioned media alone strengthens the argument for the role of activated macrophages in the progression of liver injury.

Expression was also quantified in macrophages and a reduction in protein for ARSG, GPLD1 and IFI27L2B was observed. These results indicate a role for macrophage driven inflammation in altering gene expression changes and also point to activated macrophages as a source protein production.

This is the first reported evidence for altered Arsg expression in response to inflammation. Arsg knockout in the brain has led to accumulation of cholesterol in the macrophages and purkinje cells in the cerebellum (231). Hepatic inflammation has been associated with cholesterol accumulation in Kupffer cells and their subsequent activation which is crucial to mount an inflammatory response (289). The reduction of ARSG in activated macrophages in this study is as a result of LPS treatment, it is likely that accumulation of oxLDL, an common metabolite in NASH (290) can cause the activation of macrophages and mediate the reduction of Arsg causing a subsequent lysosomal storage pathology and the development of foamy macrophages leading to a further pro-inflammatory environment. Additionally, heparan sulphate proteoglycans, the substrate for ARSG, mediate clearance of triglyceride-rich lipoproteins (291) and it can also be hypothesised that reduced ARSG in activated macrophages leads to increased uptake of lipoproteins which can further exacerbate the macrophage pathology and leads to more damage.

On activation, macrophages produce several inflammatory cytokines like TNFα, IL1, IL6 and chemokines of the CXCL and CCL family of proteins (279). Hence the reduction of IFI27L2B, an interferon stimulated gene in activated macrophages was rather unexpected.

The reduction of GPLD1 in macrophages might be related to the function of this gene in cleavage and release of glycosylphosphatidylinositol (GPI)-anchored proteins. CD55 and CD59 are GPI-anchored proteins which inhibit the formation of the complement cascade in the immune system (292). The reduction in Gpld1 expression might reduce the release of GPI-anchored CD55 and CD59 and inhibition of activation of the complement cascade (292). This
finding is in the light of another study which has examined and reported a role for the activation of the alternative immunity pathway in the development of NASH (293).

In summary (Table 6.2), this study has shown alterations in expression of GPLD1 and IFI27L2B in response to insulin stimulus and an abrogated insulin response with the co-administration of FFA and iron loading. This thesis has also demonstrated modulation of Arsg, Gpld1 and Ifi27l2b in hepatocytes and in macrophages. In hepatocytes, expression was significantly altered only in the presence of macrophage derived conditioned media and in activated macrophages gene expression was reduced for all proteins. Further experimentation will need to be performed to clarify the specific mechanisms altered by these genes in the development of injury.

Table 6.2: Summary of gene expression analysis in vitro in AML12 hepatocytes and 264.7 macrophages.

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<th>AML12 Hepatocytes</th>
<th>RAW264.7 Macrophages</th>
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<tr>
<td></td>
<td>FFA + Iron + Insulin</td>
<td>IL-6</td>
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<tr>
<td>Protein</td>
<td>RNA</td>
<td>RNA</td>
</tr>
<tr>
<td>Arsg</td>
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<td>NC</td>
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<tr>
<td>Gpld1</td>
<td>↓</td>
<td>NC</td>
</tr>
<tr>
<td>Ifi27l2b</td>
<td>↓↓</td>
<td>NC</td>
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RNA and protein expression of Arsg, Gpld1 and Ifi27l2b was quantified in in vitro in hepatocytes and macrophages. The AML12 hepatocytes were stimulated with insulin, pro-inflammatory cytokine (IL6) and LPS-CM. RAW264.7 macrophages were stimulated with LPS. The red arrows indicate upregulation and green arrows indicate downregulation in comparison to the control mice with normal liver histology. One arrow ≤ 2 fold change, two arrows > 2 fold change, four arrows ≥ 50 fold change. No change (NC). The * represents significance from Holm-Sidak’s post-hoc test at p ≤ 0.05. Arylsulfatase G (Arsg), Glycosylphosphatidylinositol phospholipase D1 (Gpld1) and Interferon alpha-inducible protein 27 like 2b was quantified (Ifi27l2b), lipopolysaccharide (LPS), lipopolysaccharide activated macrophages (LPS-CM).
Chapter 7  Final Discussion
7.1 Main findings in this thesis

NAFLD is usually considered the hepatic manifestation of the metabolic syndrome and is present in 20-30% of the western world with the incidence of NAFLD increasing at an alarming rate (294). NASH is the progressive form of NAFLD, with hepatic necroinflammation and varying degrees of fibrosis, and can develop into cirrhosis and end stage liver disease (5). At present hepatitis C virus (HCV) is the leading indication for liver transplantation (LTx) but it is predicted that by 2020, NASH will be the leading indication for LTx (4). The mechanisms that predispose NAFLD patients to the development of steatohepatitis and fibrosis are unclear but there has been an interest in the role of iron mediated oxidative stress in this progression. Steatosis in patients with the C282Y \textit{HFE} mutation has been found to be an independent risk factor for the progression of fibrosis (9) and \textit{Hfe}\textsuperscript{−/−} mice fed a HCD also developed steatohepatitis while WT mice developed simple steatosis (10). The mechanisms underlying the progressive injury are not fully understood therefore this project was undertaken with the primary aim being to utilise a transcriptomic approach to identify factors associated with the development of NASH.

Transcriptomic analysis of hepatic tissue from \textit{Hfe}\textsuperscript{−/−} mice fed chow and a HCD, found genes that were appropriately upregulated in response to a HCD such as lipid droplet proteins, \textit{Plin2} and \textit{Cidec}, which have been implicated in the development of liver steatosis (210). Genes with an unrecognised role in NASH pathogenesis were also found differentially regulated and formed the focus of this project. The genes selected were \textit{Ifi27l2b}, an interferon stimulated gene (215), and \textit{Arsg}, a lysosomal enzyme with a role in heparan sulphate degradation (221, 295) which had upregulated hepatic expression in \textit{Hfe}\textsuperscript{−/−} mice which developed steatohepatitis. Lastly we focussed on \textit{Gpld1}, an HDL-associated protein with a role in cleaving GPI-anchors (233) which was downregulated in response to a HCD in both \textit{Hfe}\textsuperscript{−/−} and WT mice.

To investigate these genes further, a model of fat and iron loading \textit{in vitro} was developed which would enable examination of underlying mechanisms of pathogenesis. A reproducible model of FFA and iron loading was developed which displayed increased expression of genes involved in \textit{de novo} lipogenesis and mitochondrial β-oxidation and reduction of expression of genes involved in fatty acid storage. These changes were also associated with an increase in expression of a pro-inflammatory cytokine indicating more severe injury with co-administration of FFA and iron. This model was also used to investigate the hepcidin signalling axis and an attenuated response to BMP6 stimulation and reduced activation of SMAD1/5/8 in
fat loaded cells was observed. This was hypothesised as a potential mechanism contributing to iron loading in NASH livers. This is the first study to have observed a blunted hepcidin response to a BMP6 stimulus and requires further investigation.

BMP and activin membrane-bound inhibitor (BAMBI) is a transmembrane protein which is known to inhibit TGF-β and BMP signalling (296, 297). In adipocytes, BAMBI knockdown has reduced the adipogenic properties of BMP4 and this was proposed to occur by reduced SMAD1/5/8 phosphorylation (298). Furthermore BAMBI expression is reduced in human fatty liver disease (299). Therefore it was hypothesized that reduced BAMBI in response to FFA loading reduces activation of SMAD1/5/8 and in turn reduces the expression of BMP6 target genes including *Hamp1* leading to increased uptake of iron. This hypothesis requires further investigation.

The candidate genes, *Arsg*, *Ifi27l2b* and *Gpld1*, found to be differentially expressed were examined in this model of FFA and iron loading in hepatocytes and in other models of chronic liver disease and an overview of the results for expression of these genes are summarised in table 7.1 and have been discussed below.

### 7.1.1 Arylsulfatase G (*Arsg*)

*Arsg* expression was increased in *Hfe*<sup>−/−</sup> mice fed HCD which developed steatohepatitis. A similar increase was seen with the development of ASH in chow fed animals. With HCD and alcohol feeding however there was no increase in *Arsg* expression. Contrary to the increase in expression seen in NASH livers, there was a reduction in expression in *Mdr2*<sup>−/−</sup> mice which developed fibrosis with an additional decline with increasing age of mice. *Arsg* expression appeared to be regulated independently of fat accumulation given the differential expression in the various models of liver injury. It was first hypothesised that the increase in ARSG, an enzyme which degrades heparan sulphate, would result in a decline in Syndecan-1 (SDC1), the predominant heparan sulphate proteoglycan (HSPG) in the liver. However, a reduction in SDC1 was not observed and this may be because ARSG may have a different HSPG substrate.

Analysis in fat loaded hepatocytes displayed a decline in *Arsg* expression and it also showed a bi-phasic expression pattern which increased at an early time point with the start of lipid accumulation in hepatocytes, followed by a decline with increasing time in culture and increasing lipid accumulation.
Table 7.1: Summary of gene expression analysis in rodents models of chronic liver disease and *in vitro* in hepatocytes and macrophages.

<table>
<thead>
<tr>
<th>A</th>
<th>NASH</th>
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<th>Mdr2&lt;sup&gt;−/−&lt;/sup&gt; (8 wk)</th>
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<td>RNA</td>
<td>Protein</td>
<td>RNA</td>
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<tr>
<td>Arsg</td>
<td>↑*</td>
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<tr>
<td>Gpld1</td>
<td>↓</td>
<td>↓*</td>
<td>↓</td>
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<tr>
<td>Ifi27l2b</td>
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<th>AML12 Hepatocytes</th>
<th>RAW264.7 Macrophages</th>
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<td>FFA + Iron</td>
<td>FFA + Iron + Insulin</td>
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<td>RNA</td>
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<td>Gpld1</td>
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RNA and protein expression of Interferon alpha-inducible protein 27 like 2b was quantified (*Ifi27l2b*), Glycosylphosphatidylinositol phospholipase D1 (*Gpld1*) and Arylsulfatase G (*Arsg*) was analysed in A) NASH, ASH and fibrotic livers and B) *in vitro* in hepatocytes and macrophages with insulin and lipopolysaccharide (LPS) stimulus. Hepatocytes were also stimulated with media from LPS activated macrophages (LPS-CM). The red arrows indicate upregulation, green arrows indicate downregulation in comparison to the control mice with normal liver histology and the dash (-) indicates that expression could not be measured. One arrow ≤ 2 fold change, two arrows ≥ 2 fold change, four arrows ≥ 50 fold change. The * represents significance from Holm-Sidak’s post-hoc test at \( p \leq 0.05 \).

*Arsg* expression in hepatocytes was also reduced after stimulation with conditioned media from activated macrophages and in the activated macrophages themselves. To the best of my knowledge, this is the first reported evidence for altered *Arsg* expression in response to FFA uptake and inflammation. *Arsg* knockout in the brain has led to a lysosomal storage disorder and the accumulation of cholesterol in macrophages and Purkinje cells in the cerebellum (231). In the liver, inflammation has been associated with cholesterol accumulation in Kupffer cells and their subsequent activation which is crucial to mount an inflammatory response (289). It can be hypothesised that macrophage activation in NASH livers could result in the downregulation of *Arsg* leading to the development of a lysosomal storage pathology and the development of foamy macrophages leading to a further pro-inflammatory environment.
Additionally, heparan sulphate proteoglycans, the substrate for Arsg, mediate clearance of triglyceride-rich lipoproteins (291) and it can also be hypothesised that reduced ARSG in hepatocytes and activated macrophages leads to increased uptake of lipoproteins which can further exacerbate the pathology.

7.1.2 Glycosylphosphatidylinositol phospholipase D1 (Gpld1)

Gpld1 expression was found reduced in the liver and serum of Hfe<sup>−/−</sup> mice fed a HCD and developed steatohepatitis. This result was contradictory to previous studies which have observed an increase in hepatic and serum GPLD1 in NASH patients (234). Latter analysis in this thesis, in livers with ASH also observed a reduction in Gpld1 expression. While Mdr2<sup>−/−</sup> mice did not display a reduction with the development of fibrosis there appeared to be an age-dependent reduction in expression. Similar results were obtained from in vitro analysis where administration of FFA resulted in a reduction of gene expression. A time course analysis also revealed a bi-phasic pattern of gene expression, like Arsg, which was increased at an early time-point at which the lipid droplet formation had just begun in the hepatocytes and was reduced at a later time point with evidence of larger lipid droplets in hepatocytes.

In vitro experiments have also suggested a role for GPLD1 in insulin signalling, and a positive correlation of GPLD1 with phospho-AKT (pAKT), a crucial modulator of insulin signalling, was demonstrated in the presence of an insulin stimulus. Gpld1 expression was reduced with FFA administration and was reduced further when treated with conditioned media from activated macrophages. Additionally, GPLD1 expression in activated macrophages itself was significantly reduced. All the analysis performed in this project has indicated a downregulation of Gpld1 expression with an external stimulus. While this reduction in gene expression was contradictory to previous findings in NASH populations, they are consistent with reports from HCC patient studies which have observed a decrease in serum GPLD1 (214). The reduction in expression was associated with an induction in proliferative capacity of cells. It could be speculated that the reduction in NASH and ASH might represent an increase in proliferative capacity in the liver, although this has not been examined in this thesis and it is yet to be determined.

GPLD1 is known to associate with high density lipoproteins and to play a role in triglyceride metabolism (233). This study however hypothesised a role for GPLD1 in NASH pathogenesis which is associated with its function as an enzyme for the cleavage of proteins with GPI-anchors. For instance, CD55 and CD59, are proteins with GPI-anchors which serve as signal
transduction molecules, and function to inhibit assembly of C3 convertase of the complement cascade (292). The downregulation of GPLD1 in this instance would reduce bio availability of CD 55 and 59 and hence inhibit the innate immune response. Increased serum levels of vascular adhesion molecule 1 (VCAM1), a GPI-anchored protein which promotes leukocyte adhesion during inflammation, was observed in patients with NAFLD (300). Conversely, the downregulation of Gpld1, as evidenced in livers with steatohepatitis, might lead to reduced VCAM1, inhibiting the inflammatory cascade. GPLD1 downregulation has also led to accumulation of GPI-anchored prion protein (PrPc) which resulted in more severe neurodegeneration (301). These findings together indicate a role for accumulating GPI-anchored proteins in the progression of injury in different organs and warrant the investigation of these and other GPI-anchored proteins in the pathogenesis of NASH.

7.1.3 Interferon, alpha-inducible protein 27 like 2B (Ifi27l2b)

The observed increase in Ifi27l2b in Hfe<sup>-/-</sup> NASH livers is consistent with the increase in expression of this gene and its transcribed protein in ASH and in Mdr2<sup>-/-</sup> mice, all of which have liver injury associated with increased inflammation, compared to the WT controls. The co-culture of hepatocytes and macrophages closely resembles the cross-talk between hepatocytes and Kupffer cells <em>in vivo</em>, which is an essential component in the development of progressive liver injury (271). A similar increase in Ifi27l2b expression was observed in hepatocytes treated with FFA and iron and additionally stimulated with conditioned media from activated macrophages.

This increase in expression of Ifi27l2b is in line with the known function of this gene as an interferon stimulated gene (ISG), which primarily responds to viral or bacterial infection to mount an immune response (215). However, recent publications from a group in China have investigated a role for interferon regulatory factors (IRFs), transcription factors mediating an interferon response, in modulating gene expression in response to nutritional and genetically induced obesity. These studies have revealed a role for IRFs in regulating energy metabolism in the liver and adipose tissue, the primary organs for lipid metabolism. One study has found a beneficial role for Irf9, where hepatic specific overexpression has improved hepatic steatosis, insulin sensitivity and inflammation (302) while the other study investigated Irf7, a master regulator of type 1 interferon response, and found that Irf7 knockout mice were resistant to diet-induced obesity, inflammation and insulin sensitivity (303). Retrospective analysis of the RNA-seq data generated in this thesis, found a significant 2-fold up-regulation of Irf7 and Irf9.
was not found in the differentially expressed dataset. The upregulation of *Irf7* might regulate *Ifi27l2b* gene expression leading to its overexpression and exacerbation of liver injury.

Similar to the aforementioned studies, evidence from this thesis has displayed a role for IFI27L2B in insulin signalling where IFI27L2B expression was positively correlated with pAKT. IFI27L2B expression appeared to reduce with the co-administration of FFA and iron despite stimulation with insulin indicating that the reduced expression may be related to the development of insulin resistance. Additionally, IFI27L2B was reduced in activated macrophages. It could be speculated that the reduced expression in macrophages might modulate hepatocyte insulin sensitivity via an inflammation driven cascade. Inflammation and insulin resistance are two key pathogenic responses associated with the development of NASH. IFI27L2B appears to sit at the cross roads of inflammation and insulin resistance to integrate the responses and drive injury. The exact mechanisms however are unknown and will require further investigation.

In all the experiments performed, iron loading itself did not have an effect on altering candidate gene expression. Iron loading in concert with FFA loading in some instances however did exacerbate the observed alterations of the candidate genes. Kupffer cell iron loading is often observed in NASH (119, 266) and investigation of the effect of iron loading did not demonstrate changes in macrophage activation status as determined by IL6 expression. Iron loading of macrophages also did not affect gene expression of *Arsg, Gpld1* or *Ifi27l2b*.

### 7.2 Potential mechanisms of *Arsg, Ifi27l2b* and *Gpld1* mediated disease pathogenesis

Given that these genes were found to be differentially expressed in the same dataset, it was tempting to investigate interactions of the genes, their transcribed proteins and substrates. Gene ontology analysis of the differentially expressed dataset generated from transcriptomics analysis failed to detect significant interactions and common pathways but when the genes were individually probed a few trends emerged and have been discussed below.

Heparan sulphate (HS), is a polysaccharide with ubiquitous expression and a myriad of functions including inflammation (230). HS is essential for immune cell transmigration and adhesion of immune cells (304) and heparan sulphates have been known to be upregulated in B-cells of the immune system in response to Type 1 IFN stimulation to mount an immune
response (305). Type 1 IFNs, IFN-κ and IFN-β (306), are known to bind to heparan sulphate, and it has been speculated that binding of IFNs to HS prevents their interaction with interferon-α receptors (IFNARs) (304). Additionally, increased heparanase, also a HSPG degrading enzyme, has led to expression of inflammatory cytokines (307, 308). Given this evidence, one other hypothesis was that increased expression of a heparan sulphate degrading enzyme, ARSG, may increase the bio-availability of IFNs and activate the Type 1 IFN signalling cascade to increase expression of ISGs like Ifi27l2b.

Many types of HSPGs exist with diverse roles and of particular interest in this project were membrane bound HSPGs, Syndecan-1 and Glypican. Syndecan-1, as mentioned previously, is known to have increased serum concentration in patients with NAFLD (255) and Glypican, is a GPI-anchored HSPG which can be shed from cellular membranes by GPLD1 (Fig 7.1). Consistent with this, there is evidence for increased serum Glypican-4 in development of insulin resistance in women with NAFLD (309). In the same study, however there was no correlation for Glypican-4 with insulin resistance in men. This suggests that there might be additional hormonal influences in the development of insulin resistance in NAFLD. Other evidence from the study in this thesis for hormonal influence in the development of NAFLD is the differential expression of Hsd3b5, a hydroxysteroid dehydrogenase, which catalyses the inactivation of testosterone (222).

This evidence supports a scenario in which the substrates of the expressed genes interact to increase the availability of bio-molecules to alter inflammatory responses and the overview of observed changes in this thesis have been outlined in Fig 7.2.

7.3 Future avenues of research

This study had initially set out to understand the mechanisms of NAFLD associated with Hfe-haemochromatosis. Transcriptomics was performed on Hfe−/− mice fed either chow or a HCD with the intention to find novel genes which underlie steatosis induction in mice with the genetic mutation. Analysis however found genes mainly altered with respect to diet rather than genotype. In order to investigate the original aim it might be pertinent to perform transcriptomics on WT mice on either diet to inform changes associated with the genetic mutation as well as diet induced alterations.
Transcriptomic analysis has identified several differentially expressed genes of which only three have been the focus of this thesis. Future work could potentially investigate some of the other genes found differentially expressed. One other interesting gene was Hydroxysteroid dehydrogenase 3β5 (Hsd3b5), a steroid metabolising enzyme which catalyses the inactivation of dihydrotestosterone (DHT) (310) and was identified as the gene with the biggest fold downregulation (13-fold) in Hfe<sup>−/−</sup> mice fed a HCD. Similarly this gene has been shown to be reduced on treatment with di(2-thylhexyl) phthalate (DEHP), a peroxisome proliferator (222, 223). The reduced expression of Hsd3b5 indicates an environment of accumulating DHT and previous studies have implicated a protective role for testosterone which reduced hepatic lipid deposition (311). Additionally, testosterone administration has inhibited hepcidin transcription (312) by interaction with BMP/SMAD signalling pathway. This might be another mechanism by which Hamp1 expression is blunted in a FFA environment and will require further examination.

**Fig 7.1: Structure of membrane bound heparan sulphate proteoglycans.** Glypican (Left) contains and N terminus globular domain which is stabilised by a disulphide bond and is membrane bound by the GPI-anchor. The HSPG can be released from the membrane by cleavage of the GPI-anchor by enzymes like GPLD1. Syndecan-1, also a HSPG does not have a GPI-anchor but can be shed from the membrane via other proteolytic enzymes (313).

Investigation of the candidate genes in *in vitro* analysis has found altered expression of these genes with various stimuli in hepatocytes as well as macrophages. In order to assess roles of these molecules it will be important to investigate the cellular source of Arsg, Gpld1 and
Ifi27l2b in liver as hepatocytes, macrophages, stellate cells or cholangiocytes. Immunohistochemical analysis of NASH livers will be essential to localise the differential expression of these proteins.

**Fig 7.2: Overview of gene expression changes with lipid loading and inflammation in Hepatocytes.** The schema presented represents the changes of gene expression in hepatocytes with A) free fatty acids (FFA) treatment alone and B) Changes in macrophages activated by lipopolysaccharide (LPS) stimulation and in hepatocytes treated with conditioned media from activated macrophages and FFA. C) Outline of the possible molecular changes associated with altered gene expression. The green arrow represents downregulation, the green arrow with two arrowheads represents a larger fold downregulation and the red arrow represents upregulation of the genes.

It will also be interesting to assess gene expression changes in other models of diet induced steatohepatitis such as methionine choline deficient diet or a “fast-food” diet to investigate if these changes are associated with the development of NASH or are specifically altered in the model used in this thesis. Conversely a leptin deficient mouse model (ob/ob mice) which eats
excessively and develops early-onset obesity could also be used to investigate if gene expression changes are modified in mice with a different genetic background.

*In vitro* knockdown and overexpression of the candidate genes has proved difficult in the AML12 cells. Use of a different cell line and other methods of gene modification will need to be investigated. Future work in this area could undertake the use of shRNA technology to enable long-term constitutive knockdown of the candidate genes.

Animal models of hepatic specific gene overexpression in a model of diet-induced obesity will be very helpful to investigate effects on the development of steatohepatitis. Adeno-associated viral delivery via tail-ven injection and oral gavage has been utilised to achieve hepatic specific transgene expression with little transduction in other organs (274, 314) and will be suited for transgene expression for this study. Apart from liver histology and serum parameters, glucose tolerance test of the mice will be essential to advise on the insulin signalling status of the genetically modified mice. It will also be interesting to investigate adipose tissue function and serum adipokines. Adipocyte and liver cross-talk in the development of liver injury is becoming evident (315-317) and it will be important to assess the development of steatosis in the liver and hypertrophy of adipose tissue. As discussed, the role of macrophages in foam cell formation and activation appear to play a role in the development of NASH and should also be assessed. While there was no direct evidence for the role of $\text{Hfe}^{-}$ or iron loading for alteration of gene expression analysis some genes have been differentially regulated in $\text{Hfe}^{-}$ mice in comparison to WT mice in this study. Therefore the assessment of iron loading and specific localisation in livers will be very useful. The blunted activation of SMAD1/5/8 in response to fat loading and hence reduced hepcidin expression, in the development of NAFLD and associated iron load will also need to be confirmed *in vivo*.

**Hypotheses for future work:**

1) Reduced BAMBI expression in response to FFA loading diminishes activation of SMAD1/5/8 and *Hamp1* expression, facilitating increased uptake of iron.

2) HSPG abundance and sulphation status are altered in diet-induced obesity and sulphation status will be a beneficial tool in predicting progression to NASH.

3) Reduced hepatic and serum GPLD1 drives a proliferative phenotype and are predictors of HCC.

4) Insulin signalling can be altered by altering expression of interferon stimulated gene *Ifi27l2b*. 
7.4 Conclusion

The work in this thesis has enabled the identification of novel genes with an unknown role in the development of Hfe-associated non-alcoholic fatty liver disease. This study has also successfully developed a model of FFA and iron loading in hepatocytes which will enable further research in this field. To the best of my knowledge, this has been the first study to find a role for altered SMAD1/5/8 signalling in FFA loaded hepatocytes, which could be a mechanism by which iron loading is exacerbated in fatty liver disease. Additionally, the work in this thesis has opened a new avenue of research for the investigation of the genes Arsg and Ifi27l2b in the development of NASH and provided evidence for bi-phasic expression of soluble protein GPLD1 which could be exploited as a bio-marker for the prediction of severity of liver injury. This thesis has laid the foundation and outlined an extensive plan for future work in this area. Interrogation of these candidates might reveal new mechanisms in the development of fatty liver disease and may indicate new strategies for therapeutic intervention.
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Appendix 1: Buffers for SDS-PAGE and agarose gel electrophoresis

Table 1: Components of a 10% resolving gel (20 ml) used for SDS-PAGE

<table>
<thead>
<tr>
<th>Components</th>
<th>Required volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5M Tris-HCl pH 8.8</td>
<td>5 ml</td>
</tr>
<tr>
<td>Deionised water</td>
<td>8.2 ml</td>
</tr>
<tr>
<td>30 % Bisacrylamide</td>
<td>8.4 ml</td>
</tr>
<tr>
<td>20 % Sodium dodecyl sulfate</td>
<td>100 µl</td>
</tr>
<tr>
<td>10 % Ammonium persulfate</td>
<td>100 µl</td>
</tr>
<tr>
<td>Tetraethylmethylenediamine (TEMED)</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Table 2: Components of a 4% stacking gel (10 ml) used for SDS-PAGE

<table>
<thead>
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<th>Components</th>
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</thead>
<tbody>
<tr>
<td>0.5M Tris-HCl pH 6.8</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>Deionised water</td>
<td>4.1 ml</td>
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<td>30 % Bisacrylamide</td>
<td>1.3 ml</td>
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<tr>
<td>20 % Sodium dodecyl sulfate</td>
<td>50 µl</td>
</tr>
<tr>
<td>10 % Ammonium persulfate</td>
<td>50 µl</td>
</tr>
<tr>
<td>Tetraethylmethylenediamine (TEMED)</td>
<td>5 µl</td>
</tr>
</tbody>
</table>
Table 3: Buffers recipes for agarose gel electrophoresis and western blot experiments

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td>TAE (50X concentrate)</td>
<td>242 g Trizma Base</td>
</tr>
<tr>
<td></td>
<td>57.1 mL glacial acetic acid</td>
</tr>
<tr>
<td></td>
<td>18.61 g EDTA</td>
</tr>
<tr>
<td></td>
<td>1000 mL</td>
</tr>
<tr>
<td>Tris Buffered Saline (TBS) 10X Stock</td>
<td>Tris-HCl 48.4 g</td>
</tr>
<tr>
<td>pH – 7.6</td>
<td>Trizma Base 11.12 g</td>
</tr>
<tr>
<td></td>
<td>NaCl 160 g</td>
</tr>
<tr>
<td></td>
<td>2000 mL</td>
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<tr>
<td>TBS-0.1%Tween 20 (TBS/T)</td>
<td>100 mL 10x TBS stock</td>
</tr>
<tr>
<td></td>
<td>1 mL Tween 20</td>
</tr>
<tr>
<td></td>
<td>1000 mL</td>
</tr>
<tr>
<td>Tris Glycine SDS (TGS) 10X Stock</td>
<td>Trizma Base 30.3 g</td>
</tr>
<tr>
<td></td>
<td>Glycine 144.0 g</td>
</tr>
<tr>
<td></td>
<td>SDS 10 g</td>
</tr>
<tr>
<td></td>
<td>1000 ml</td>
</tr>
<tr>
<td>Transfer Buffer (TG) 10X Stock</td>
<td>Trizma Base 30.3 g</td>
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<tr>
<td></td>
<td>Glycine 144.0 g</td>
</tr>
<tr>
<td></td>
<td>1000 mL</td>
</tr>
<tr>
<td>Western Blot Resolving Gel Buffer 1.5M Tris-HCl</td>
<td>Tris-HCl 7.38 g</td>
</tr>
<tr>
<td>pH 8.8</td>
<td>Trizma Base 30.78 g</td>
</tr>
<tr>
<td></td>
<td>200 ml</td>
</tr>
<tr>
<td>Western Blot Stacking Gel Buffer 0.5 M Tris-HCl</td>
<td>Trizma Base 12 g</td>
</tr>
<tr>
<td>pH 6.8</td>
<td>Deionized water 150 mL</td>
</tr>
<tr>
<td></td>
<td>200 ml</td>
</tr>
<tr>
<td>5% Blocking Buffer for Western Blot</td>
<td>5 g skim milk powder</td>
</tr>
<tr>
<td></td>
<td>100 mL 1x TBS/T</td>
</tr>
</tbody>
</table>
Appendix 2: Diet composition for animal feeding

Table 1: Macronutrient components of diets utilised in this project

<table>
<thead>
<tr>
<th>Dietary component</th>
<th>Chow</th>
<th>High Calorie Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein, % weight</td>
<td>17.4</td>
<td>19.36</td>
</tr>
<tr>
<td>Carbohydrate, % weight</td>
<td>42</td>
<td>52.5</td>
</tr>
<tr>
<td>Total Fat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% weight</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>% calories</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>Total calories kcal/g</td>
<td>3.30</td>
<td>4.78</td>
</tr>
<tr>
<td>Ingredients (g/Kg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Casein</td>
<td>0</td>
<td>200</td>
</tr>
<tr>
<td>Sucrose</td>
<td>7</td>
<td>405</td>
</tr>
<tr>
<td>Canola oil</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Cocoa butter, g/Kg</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>Hydrogenated vegetable oil</td>
<td>0</td>
<td>131</td>
</tr>
<tr>
<td>Cellulose</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>413</td>
<td>50</td>
</tr>
<tr>
<td>Dextrinised starch</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

HCD; SF03-020 from Speciality feeds, Glen Forrest, WA
Appendix 3: Phred quality score

Table 1: Phred quality scores with the associated probability that the base is called incorrectly

<table>
<thead>
<tr>
<th>Phred Quality score</th>
<th>Probability that the base is called wrong</th>
<th>Accuracy of the base call</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1 in 100</td>
<td>99%</td>
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<tr>
<td>30</td>
<td>1 in 1,000</td>
<td>99.9%</td>
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<tr>
<td>40</td>
<td>1 in 10,000</td>
<td>99.99%</td>
</tr>
<tr>
<td>50</td>
<td>1 in 100,000</td>
<td>99.999%</td>
</tr>
</tbody>
</table>

Phred is a base-calling program for DNA sequence reads. The program analyses DNA sequence chromatogram files and assigns quality scores or ‘Phred scores’ for each base call (208).
Appendix 4: Plasmid Maps

Figure 1: Maps of plasmid vectors used for gene overexpression studies. A) Arsg and Gpld1 cDNA were cloned in the PCV/Kan-Neo vector within EcoR1 and Not1 restriction sites under the control of the cytomegalovirus (CMV) promoter (orange). The plasmid also carried a kanamycin resistance gene (blue) to enable bacterial selection. B) Ifi27l2b (yellow) was cloned with a HA-tag at the C-terminus in the pcDNA3.1/Zeo (+) vector and was a gift from Dr Liao, Taiwan. The gene was under the control of the CMV promoter (orange) and carried the Ampicillin resistance gene for bacterial selection.
Figure 2: Plasmid map of the adeno-associated viral (AAV) vector. The AAV vector has the AAV2 inverted terminal repeats to enable viral genome replication and can be used for transfection in vivo. It has a cytomegalovirus (CMV) promoter and an ampicillin resistance gene to enable bacterial selection. EcoR1 and SalI restriction enzymes located in the multiple cloning sites and were used as cloning sites but due to problems with cloning, XhoI (not depicted in the map) was used instead.