A Global Approach to Dissecting Lipid-induced Impairment of Liver Gluconeogenesis

by

Bradley J. Hayward
BForensicSc (Hons)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Deakin University
March, 2013
I am the author of the thesis entitled *A Global Approach to Dissecting Lipid-induced Impairment of Liver Gluconeogenesis*

submitted for the degree of **Doctor of Philosophy**

This thesis may be made available for consultation, loan and limited copying in accordance with the Copyright Act 1968.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: .................................................................

(Please Print)

Signed: .................................................................

Signature Redacted by Library

Date: .................................................................

5/3/2013
I certify that the thesis entitled A Global Approach to Dissecting Lipid-induced Impairment of Liver Gluconeogenesis

submitted for the degree of Doctor of Philosophy

is the result of my own work and that where reference is made to the work of others, due acknowledgment is given.

I also certify that any material in the thesis which has been accepted for a degree or diploma by any university or institution is identified in the text.

'I certify that I am the student named below and that the information provided in the form is correct'

Full Name: .................................................................
(Please Print) BRADLEY JAMES HAYWARD

Signed: .................................................................

Signature Redacted by Library

Date: .................................................................

5.3.2013
Abstract

Given the increasing worldwide obesity epidemic, there exists a need for development of alternative therapies for lipid-induced insulin resistance and associated increases to hepatic glucose production – a key driver of type 2 diabetes (T2D). The complexity of insulin resistance hinders attempts to find therapies that are effective on a population scale with minimal side effects, and remain viable treatments over the lifetime of a patient. We propose that embracing this complexity by using unbiased global approaches to characterise both the induction (“diseased” state) and reversal (“recovered from disease” state) of lipid-induced alterations to hepatic glucose production will prove effective for the identification of additional targets and therapies. Modelling both the induction and reversal of such a state is a relevant representation of route to recovery from disease that an individual faces. Microarray technology provides two key methods to investigate such a model. Firstly, the identification of a small subset of genes (gene expression signature; GES) with the best ability to discriminate between “diseased” and “recovered from disease” states. Identification of these genes would enable GES-based small molecule screens to find therapeutics that can match an individual’s GES profile. We propose that this personalised medicine approach will allow for identification of therapies with optimised efficacy. Secondly, by identifying regulated signalling and metabolic pathways between the “diseased” and “recovered from disease” states, we may discover novel metabolic regulation occurring in parallel with the disease. Such a finding would provide additional targets for the development of treatments for patients with T2D.
Cellular insulin resistance and elevated basal glucose production were induced in rat FAO hepatoma cells treated with 0.075 mM palmitate (PA-treated) for 48 h. In this in vitro model, insulin resistance was not reversed using a series of known anti-diabetes drugs including biguanides, salicylates, thiazolidinidiones or the traditional Chinese herbal extract berberine, in contrast to the effects of these drugs in other cell types where they have been shown to improve insulin sensitivity. However, the reversal of elevated glucose production was achieved via combination treatment with 0.25 mM metformin and 2 mM sodium salicylate (PAMN-treated) added in the final 24 h of PA-treatment. Combination treatment was a deliberate feature of the model – making the GES characteristic of a multi-target approach to reverse dysregulated glucose production, rather than characterising the effects of one specific anti-diabetic. We propose that this modelling approach will enhance the ability to identify optimal therapies during drug screening.

cDNA microarrays were utilised to profile the global transcriptional changes associated with the onset and reversal of PA-induced dysregulated glucose production in the cells. Diagonal linear discriminate analysis (DLDA) of the microarray dataset identified a set of 7 genes that best discriminated between the induction and reversal of PA-induced dysregulated glucose production. The gene set included annexin a3 (Anxa3); glutathione-s-transferase m-3 (Gstm3); phospholipase A2, group 1b (Pla2g1b); ras-related protein Rab-33a (Rab33a); serine peptidase inhibitor, clade A, member 5 (Serpina5); solute carrier family 2, member 2 (Slc2a2/GLUT2) and stannin (Snn). The gene expression levels of these genes were not reproducibly validated via RT-PCR, therefore preventing the development of a PA-based GES at this time. The expression levels of each GES gene was determined
in a human lymphocyte dataset from the San Antonio Family Heart Study ($n = 1,240$), and $Anxa3$ gene expression levels were positively and negatively correlated with total serum triglyceride and fasting plasma glucose levels, respectively. After using linear regression analysis to adjust for age, sex and BMI, $Anxa3$ was not found to be an independent predictor of either of these phenotypes.

Gene set enrichment analysis of the microarray dataset demonstrated that the pathways being regulated at the transcriptional level were different in PA-induced dysregulated glucose production compared with those involved in the reversal with PAMN. The effects of PA were characterised by transcriptional regulation of cell cycle, immune function and inositol phosphate metabolism – the latter of particular relevance to insulin signalling. In contrast, PAMN-treatment was characterised by a significant decrease in the metabolic pathways regulating the synthesis of cholesterol and bile acids from acetyl-CoA, which have proposed roles in regulating fatty acid uptake, insulin sensitivity and hepatic glucose production, respectively.

A global mass spectroscopy-based lipidomic profile was generated to investigate the effects of transcriptional regulation on lipid metabolism. PAMN-treatment was characterised by decreased levels of multiple diacylglycerols (DAGs), triacylglycerols (TAGs) and cholesterol ester species that contained at least one C18:1 side chain in parallel with decreased glucose production following PAMN-treatment. Given the role these unsaturated species have been suggested to play in activation of PKC isoforms (specifically PKCε in the liver), this finding may indicate a role for PKCε activation in this model. The total levels of intracellular or secreted cholesterol and bile acids between cell treatment groups were not different; however, whether the
transcriptional changes described above indicate altered metabolic flux through these pathways remains to be confirmed. A comprehensive analysis of the phosphatidylinositol (PI) content of vehicle-, PA- and PAMN-treated cells identified PI 38:5 as a candidate target mediating the effects of PA-treatment on hepatic glucose production, as it was decreased in parallel with insulin sensitivity. Further analysis revealed the major identity of the side chains of PI 38:5 as oleic acid (C18:1) and arachidonic acid (C20:4). Currently, the contribution of specific PI species in insulin signalling and hepatic glucose production remains unresolved.

Taken together, the results implicate the rate of acetyl-CoA to cholesterol synthesis as a possible key regulator of hepatic glucose production. There is also evidence for the identity of PI side chains to modulate insulin signalling, providing an entirely new class of lipid targets for the reversal of lipid-induced insulin resistance and/or elevated glucose production. In addition, while development of a PA-based GES was unsuccessful at this time using DLDA for gene identification, the development of such a GES remains possible using alternative Bayesian statistical approaches. Candidate gene identification via DLDA did identify both Anxa3 and Pla2g1b, one of which (Pla2g1b) has been previously implicated in lipid-induced dysregulation of hepatic glucose production. Therefore, this study has implicated the following three metabolic targets in regulation of hepatic glucose production – acetyl-CoA to cholesterol metabolism, lipid species with C18:1 side chains, and PI 38:5. These potentially novel mediators of hepatic glucose production may therefore provide targets for development of therapeutic agents for T2D. Characterising both the induction and reversal of PA-induced dysregulated glucose production identified these metabolic targets. This approach may therefore have similar utility in future
studies identifying novel therapeutic targets for alternative disease states that can be modelled \textit{in vitro}.
Acknowledgements

First and foremost I would like to thank both of my supervisors- Professor Ken Walder and Dr Nicky Konstantopoulos for their ongoing support and guidance over the last three and a half years. Both of you have provided assistance in reading drafts, sitting through practice presentations and have always made yourself available to discuss my work and where we should head next. Ken, I fondly recall you accompanying me on a whirlwind trip to the EASD conference in Lisbon, and the experience really opened my eyes on the opportunities such a scientific career brings. Nicky, your enthusiasm for science has been an inspiration to me during the completion of my studies. You are always keen to hear about the latest results (even while you’re supposed to be on leave!), and I am incredibly grateful of the time and effort you have put into helping me produce this thesis.

I also owe thanks to my original associate supervisors Dr Juan Carlos Molero and Associate Professor Jeremy Jowett. Juan, your guidance in setting up my original cell model is greatly appreciated. Despite moving to RMIT at the end of my second year, I always valued our intellectually stimulating discussions. I will always appreciate Jeremy’s willingness to make time to discuss my project and our discussions about the human lymphocyte data. Jeremy’s passing during the final year of my thesis brought great sadness; I will always fondly recall our scientific discussions and his genuine interest in how my work was progressing.
Dr Carsten Schmitz-Peiffer from the Garvan Institute of Medical Research was one of the greatest external influences on my studies. Our discussions on pathway regulation and cholesterol metabolism was a driving force in shaping the second half of this thesis, and I look forward to continuing our discussions in the future. To both Dr Kelly Windmill and Andrew Sanigorski, your assistance in setting up the microarray studies and helping in the interpretation of the data was invaluable. Professor Peter Meikle and Ms Jacqui Weir from the Baker IDI Heart and Diabetes Institute both provided assistance in performing the lipidomic profiling performed as part of my research, and your technical assistance is greatly appreciated. Further, to Professor Andy Sinclair, your guidance on lipid metabolism was invaluable in interpreting the results of the lipidomic profiling studies, and your friendship and guidance throughout my studies has been greatly appreciated.

To the rest of the MRU staff, I am grateful for your friendship over the last three and a half years. Briana Spolding, Tim Connor, Sheree Martin, Shona Morrison, Courtney Swinton, Rachel Abela, Dr Kathryn Aston-Mourney, Natasha McRae and Jane Hosking, – you have all provided technical assistance in the laboratory or sat through a practice presentation of mine at some stage over the last few years, and your friendship and humour made working at the MRU a fantastic experience. I would also like to acknowledge the camaraderie shared by my fellow PhD students – Vidhi Gaur, Juliane Czeczor, Dan Fraher, Simon Bond and Samaneh Ghasemi Fard – and I wish them all the best for their future careers.

Finally, I could not have successfully completed my studies without the ongoing and unconditional support of my family. To my parents Sue and Ross, you have always
encouraged me to strive to achieve my very best and I am incredibly fortunate to have grown up in such a supportive family. To my brothers Ben and Josh, thanks for the support and occasional distraction when it was needed. I’m looking forward to the next few years when I’ll be seeing you both complete your PhD studies as well. I guess Mum and Dad must have done something right! And to my wonderful partner Megan, your support has meant the world to me. Thank you for putting up with late nights and weekends spent at the lab. This year has been a crazy one filled with exams and thesis writing, but we made it. I’m looking forward to seeing what the future has in store for us.
# Table of Contents

## CHAPTER 1: INTRODUCTION .................................................................1

1.1. **Complexity of Diabetes Mellitus** .................................................1  
1.1.1. *Prevalence and cost of the worldwide diabetes burden* ..........1  

1.2. **Diagnosis and Treatment of Type 2 Diabetes (T2D)** ......................2  
1.2.1. *Metabolic staging of T2D* .........................................................2  
1.2.2. *Diagnosis of T2D* .................................................................4  
1.2.3. *Current treatments for individuals with T2D* ......................5  
1.2.4. *Problems and adverse effects of current drug therapies* .........8  

1.3. **Complexity of T2D and Insulin Resistance Subtypes** ....................9  
1.3.1. *Insulin resistance subtypes* .....................................................9  
1.3.2. *The rising worldwide prevalence of obesity* .....................12  

1.4. **Obesity** .................................................................................13  
1.4.1. *Free fatty acids* .................................................................13  
1.4.2. *Effect of dietary free fatty acid composition on insulin signalling* .....14  
1.4.3. *Normal metabolic interplay between carbohydrate and fatty acid metabolism/oxidation* .........................................................16  
1.4.4. *Effects of saturated free fatty acids on insulin sensitivity in skeletal muscle* .................................................................17  
1.4.4.1. *Ceramide accumulation and the atypical protein kinase C-zeta (PKCζ)* .................................................................18  
1.4.4.2. *Diacylglycerol (DAG) accumulation and Protein Kinase C activation* .................................................................19  
1.4.4.3. *Reactive oxygen species (ROS) accumulation and c-Jun NH2-terminal kinase (JNK) activation* .................................................................20  
1.4.5. *Effects of free fatty acids on insulin sensitivity in the liver* ........21  
1.4.5.1. *DAG accumulation and Protein Kinase C-epsilon (PKCe)* ..........22  
1.4.5.2. *Hepatic ceramide accumulation* ...........................................23  
1.4.5.3. *Reactive oxygen species (ROS) accumulation and JNK activation* .................................................................24  
1.4.5.4. *Induction of the pro-inflammatory NF-κB pathway* ..........25  
1.4.6. *Insulin independent effects on basal glucose production* ........25
1.4.6.1. Acetyl-CoA and pyruvate carboxylase ............................................. 26
1.4.6.2. p38 MAP-kinase activation by fatty acids ........................................ 26
1.4.7. Lack of effect of free fatty acids on insulin sensitivity in adipose tissue ...................................................................................................... 27
1.5. Previous approaches for characterising insulin resistance ..................... 28
1.5.1. Classical single target-based approaches ............................................ 28
1.5.2. Endpoint-based approaches ................................................................. 29
1.5.3. ‘Omics’ approaches .............................................................................. 29
1.5.4. Genomics-based approaches ................................................................. 30
1.6. Gene expression signatures ........................................................................ 32
1.6.1. Gene expression signatures as a diagnostic tool .................................... 32
1.7. Overview of GES development .................................................................. 34
1.7.1. Characterising insulin resistance in vitro ............................................. 36
1.7.2. Identification of putative GES genes .................................................... 37
1.7.3. Applications of GES in the development of personalised treatment for individuals with T2D ...................................................................................................... 39
1.7.3.1. Using GES to stratify patients with T2D ............................................ 39
1.7.3.2. Using the GES to develop “targeted” therapies .................................. 40
1.7.4. Proof of principle: Inflammation-induced cellular “insulin resistance” 41
1.7.5. Identification of PA-derived GES from liver cells .................................. 43
1.8. Summary ....................................................................................................... 44
1.9. Aims .............................................................................................................. 45

CHAPTER 2 – IN VITRO MODEL DEVELOPMENT OF HYPERLIPIDEMIA-INDUCED DYSREGULATION OF GLUCOSE HOMEOSTASIS ................................................................. 46

2.1. Introduction ..................................................................................................... 46
2.1.1. Modelling dysregulated glucose homeostasis ........................................ 46
2.1.2. Reversing dysregulated glucose homeostasis ........................................ 47
2.2. Methods ........................................................................................................... 52
2.2.1. Cell culture ............................................................................................ 52
2.2.2. Cell treatments ..................................................................................... 52
2.2.3. Measurement of glucose production in FAO hepatoma cells ............... 53
2.2.4. Cellular viability and cytotoxicity measurements in response to lipid and drug treatments ................................................................. 54
2.2.5. Statistical analysis ............................................................................ 55
2.3. RESULTS ..................................................................................................... 57
  2.3.1. Effects of PA- and LA-treatment on glucose production in FAO hepatoma cells ................................................................. 57
  2.3.2. Reversing PA dysregulation of glucose production in FAO hepatoma cells ............................................................... 64
    2.3.2.1. Salicylates ......................................................................................... 68
    2.3.2.2. Biguanides ......................................................................................... 69
    2.3.2.3. Thiazolidinediones ............................................................................ 70
    2.3.2.4. Berberine .......................................................................................... 70
  2.3.4. Optimising combination treatment to reverse PA dysregulation of glucose production in FAO hepatoma cells .................................. 72
  2.3.5. Metformin and sodium salicylate reverse PA dysregulation of glucose production in FAO hepatoma cells ........................................ 74
2.4. DISCUSSION ................................................................................................. 78

CHAPTER 3 – IDENTIFICATION OF A GES THAT DISCRIMINATES BETWEEN DYSREGULATED HEPATIC GLUCOSE PRODUCTION (PA-TREATED) AND RESTORED, HEALTHY HEPATIC GLUCOSE PRODUCTION (PAMN-TREATED) ................................................... 85

3.1. INTRODUCTION .............................................................................................. 85
3.2. METHODS ...................................................................................................... 89
  3.2.1. Selection of samples for microarray analysis ...................................... 89
  3.2.2. RNA extractions and microarrays ...................................................... 91
  3.2.3. Diagonal Linear Discriminate Analysis (DLDA) for identification of GES genes ................................................................. 92
  3.2.4. RNA extractions and RT-PCR validation of GES genes .................... 93
  3.2.5. Statistical analysis ............................................................................... 94
3.3. RESULTS ....................................................................................................... 96
  3.3.1. DLDA identified the most discriminating genes between PA- and PAMN-treated cells ................................................................. 96
3.3.2. Validation of the potential PA-based GES genes using RT-PCR .......... 100
3.3.3. Human phenotypic profiling of the candidate gene Anxa3 ................ 104
3.4. DISCUSSION ......................................................................................................... 106

CHAPTER 4 – GLOBAL GENE EXPRESSION PROFILING OF PA-INDUCED
DYSREGULATION OF HEPATIC GLUCOSE PRODUCTION, AND RESTORATION WITH
METFORMIN AND SODIUM SALICYLATE ........................................................................ 111

4.1. INTRODUCTION ........................................................................................................ 111
4.2. METHODS ................................................................................................................ 113
  4.2.1. Gene Set Enrichment Analysis (GSEA) using DAVID ..................... 113
4.3. RESULTS ................................................................................................................... 115
  4.3.1. Gene pathways induced with PA-mediated dysregulation of glucose
production ....................................................................................................................... 115
  4.3.1.1. Gene set enrichment analysis between vehicle- and PA-treated FAO
cells identified multiple regulatory pathways in cell cycle regulation .......... 117
  4.3.1.2. Gene set enrichment analysis between vehicle and PA-treated FAO
cells identified multiple regulatory pathways in immune function and
inflammation .................................................................................................................. 118
  4.3.1.3. Gene set enrichment analysis between vehicle and PA-treated FAO
cells identified regulation of inositol phosphate metabolic pathways ......... 119
  4.3.2. Regulated gene pathways induced with PAMN-mediated reversal of
PA-induced dysregulation of glucose production ..................................................... 120
  4.3.2.1. Gene set enrichment analysis between PA-and PAMN-treated FAO
cells identified multiple regulatory pathways of both the fatty acid and amino
acid biosynthetic pathways ......................................................................................... 122
  4.3.2.2. Gene set enrichment analysis between PA-and PAMN-treated FAO
treated FAO cells identified changes in the cholesterol and bile acid
synthetic pathways ....................................................................................................... 125
  4.3.2.3. Gene set enrichment analysis between PA-and PAMN-treated FAO
cells identified regulation of multiple lipid metabolic pathways .................. 129
  4.3.2.4. Gene set enrichment analysis between PA-and PAMN-treated FAO
cells identified transcriptional regulation of PPAR signalling ................... 131
4.3.2.5. Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified transcriptional regulation of multiple pathways involving immune function. 133

4.3.2.6. Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified transcriptional regulation of mediators of oxidative stress. 134

4.4. DISCUSSION ................................................................................................... 136

CHAPTER 5 – PA-INDUCED ALTERATIONS TO THE GLOBAL LIPIDOMIC PROFILE IN FAO HEPATOMA CELLS ................................................................. 144

5.1. INTRODUCTION ............................................................................................... 144

5.2.1. Lipidomic profiling of FAO cells following PA- and PAMN-treatment 147

5.2.2. Analysis of excreted cholesterol levels in conditioned media .......... 149

5.2.3. Measurement of excreted and intracellular total bile acids .......... 150

5.2.4. RNA extractions and RT-PCR analysis of gluconeogenetic genes ...... 151

5.2.5. Statistical analysis .............................................................................. 152

5.3. RESULTS ........................................................................................................ 153

5.3.1. Regulation of the TAG lipid profile by palmitate, metformin and sodium salicylate ................................................................. 154

5.3.2. Regulation of the DAG lipid profile by palmitate, metformin and sodium salicylate ................................................................. 156

5.3.3. Regulation of the cholesterol lipid profile by palmitate, metformin and sodium salicylate ................................................................. 158

5.3.4.1. Excreted cholesterol was detected at very low levels and was not regulated by either PA- or PAMN-treatment............................................. 160

5.3.5. Total bile acids were not regulated by PA- or PAMN-treatment in FAO hepatoma cells ................................................................. 162

5.3.6. Modest regulation of the ceramide lipid profile by PA- and PAMN-treatment ................................................................................................................................. 163

5.3.7. Modest regulation of the sphingomyelin lipid profile by PA- and PAMN-treatment ................................................................................................................................. 166

5.3.8. Regulation of the phosphatidylinositol lipid profile by palmitate, metformin and sodium salicylate ........................................................................................................ 168
5.3.8.1. Characterisation of lipid side chains on phosphatidylinositol 38:5 171
5.3.9. Increased lipid levels for species containing palmitate side chains was not common to all measured species.................................................................173
5.3.10. PA-treatment was not associated with changes to the gluconeogenetic genes Pepck or G6Pase ..................................................................................... 176
5.4. DISCUSSION ........................................................................................................ 178

CHAPTER 6 –CONCLUSIONS AND FUTURE DIRECTIONS....................................... 186
6.1. IDENTIFICATION OF CANDIDATE GES GENES FOR CONFIRMATION IN A HUMAN COHORT .187
6.2. THE ROLE OF ANXA3 IN DYSREGULATED GLUCOSE PRODUCTION AND WHOLE BODY INSULIN RESISTANCE.................................................................................................................. 189
6.3. THE CONTRIBUTION OF THE CHOLESTEROL METABOLISM PATHWAY TO WHOLE BODY GLUCOSE METABOLISM .................................................................................................................. 191
6.4. CONFIRMING THE ROLE OF PI SIDE CHAINS IN THE INSULIN SIGNALLING CASCADE .......... 193
6.5. SUMMARY ..................................................................................................... 194

APPENDIX 1 – IN VITRO MODEL DEVELOPMENT OF PA-INDUCED DYSREGULATION OF GLUCOSE HOMEOSTASIS IN MUSCLE CELL LINES. ............................................. 198
A1.1. INTRODUCTION ............................................................................................... 198
A1.2. METHODS ..................................................................................................... 199
 a1.2.1. Cell culture .......................................................................................... 199
 a1.2.2. Cell treatments ................................................................................... 200
 a1.2.3. Glycogen synthesis measurement in C2C12 myotubes ...................... 200
 a1.2.4. 2-Deoxyglucose uptake in L6 myotubes ............................................. 201
 a1.2.5. HA-GLUT4 translocation measurement in stable L6-GLUT4 myoblasts .................................................................................................................. 201
 a1.2.6. Statistical analysis .............................................................................. 203
A1.3. RESULTS ....................................................................................................... 205
 a1.3.1. Effects of PA-treatment on basal and insulin-stimulated glycogen synthesis in C2C12 myotubes .................................................................................................................. 205

XIV
a1.3.2. Effects of PA-treatment on basal and insulin-stimulated glucose uptake in L6 myotubes

a1.3.3. Effects of PA-treatment on basal and insulin-stimulated HA-GLUT4 translocation in stable HA-GLUT4-L6 myoblasts

A1.4. DISCUSSION

APPENDIX 2 – REVERSAL OF 48 H PA-TREATMENT INDUCED HIGH GLUCOSE PRODUCTION IN FAO HEPATOMA CELLS BY COMBINATION TREATMENT USING METFORMIN AND SODIUM SALICYLATE

APPENDIX 3 – GENES IDENTIFIED THROUGH DLDA

APPENDIX 4 – KEGG PATHWAYS IDENTIFIED THROUGH GSEA AS BEING DIFFERENTIALLY REGULATED BETWEEN VEHICLE- AND PA-TREATED CELLS

APPENDIX 5 – KEGG PATHWAYS IDENTIFIED THROUGH GSEA AS BEING DIFFERENTIALLY REGULATED BETWEEN PA- AND PA WITH METFORMIN AND SODIUM SALICYLATE-TREATED CELLS

APPENDIX 6 – SIDE CHAIN DETERMINATION OF PI 38:5 USING THE PRODUCTION ION SCAN APPLICATION OF LIQUID CHROMATOGRAPHY, ELECTROSPRAY IONISATION-TANDEM MASS SPECTROMETRY (LC ESI-MS/MS) ANALYSIS OF INTRACELLULAR LIPID FRACTIONS

REFERENCES
List of Figures

FIGURE 1.1: METABOLIC STAGING OF T2D. ........................................................... 3
FIGURE 1.2: THE MULTIFACTORIAL NATURE OF T2D............................................. 10
FIGURE 1.3: SYNTHESIS AND OXIDATION OF INTRACELLULAR FATTY ACIDS. ...... 16
FIGURE 1.4: INTERPLAY BETWEEN HEPATIC FATTY ACID UTILISATION AND GLUCOSE PRODUCTION. ..................................................................................... 21
FIGURE 1.5: DEVELOPMENT PROCEDURE FOR A GES FOR INSULIN RESISTANCE. .. 34
FIGURE 1.6: DISTINCT CELLULAR STATES REQUIRED FOR DEVELOPMENT OF A GES FOR INSULIN RESISTANT SUBTYPES. ................................................................. 36
FIGURE 1.7: STRATIFICATION OF PATIENTS ACCORDING TO THEIR SIMILARITIES TO THE GES MODELS OF INSULIN RESISTANCE ................................................. 40
FIGURE 2.1: SCHEMATIC REPRESENTATION OF THE 48 H TREATMENT REGIME FOR THE INDUCTION AND REVERSAL OF PA INDUCED DYSREGULATION OF GLUCOSE PRODUCTION. ................................................................. 56
FIGURE 2.2: DYSREGULATION OF GLUCOSE PRODUCTION BY 24 H TREATMENT WITH PA OR LINOLEATE IN FAO HEPATOMA CELLS. ............................................... 59
FIGURE 2.3: DYSREGULATION OF GLUCOSE PRODUCTION AND CELLULAR VIABILITY BY 48 H TREATMENT WITH PA OR LINOLEATE IN FAO HEPATOMA CELLS. ............. 62
FIGURE 2.4: INCREASED CYTOTOXICITY BY 48 H TREATMENT WITH 0.3 MM PA IN FAO HEPATOMA CELLS. .................................................................................. 63
FIGURE 2.5: REVERSAL OF PA-INDUCED GLUCOSE DYSREGULATION BY ANTI-DIABETIC AGENTS IN FAO HEPATOMA CELLS....................................................... 67
FIGURE 2.6: COMBINATION REVERSAL OF PA-INDUCED GLUCOSE DYSREGULATION USING 0.25 MM METFORMIN AND 2 MM SODIUM SALICYLATE IN FAO HEPATOMA CELLS........................................................................................................... 77
FIGURE 3.1: FLOWCHART FOR GES DEVELOPMENT............................................. 89
FIGURE 3.2: GLUCOSE PRODUCTION FOR SELECTED MICROARRAY SAMPLES. ...... 90
FIGURE 3.3: CANDIDATE GENE EXPRESSION LEVELS AS MEASURED VIA MICROARRAY ANALYSIS. ................................................................. 98

FIGURE 3.4: CANDIDATE GES GENE EXPRESSION LEVELS AS MEASURED VIA RT-PCR. ................................................................................. 103

FIGURE 4.1: FLOWCHART FOR IDENTIFICATION OF REGULATED PATHWAY USING DAVID. ................................................................. 113

FIGURE 4.2: PAMN-INDUCED CHANGES IN METABOLIC PATHWAYS REGULATING ACETYL-COA METABOLISM. ........................................ 124

FIGURE 4.3: BIOSYNTHESIS PATHWAYS IDENTIFIED AS BEING REGULATED AT THE TRANSCRIPTIONAL LEVEL BETWEEN PA-AND PAMN-TREATED CELLS VIA GSEA. 127

FIGURE 4.4: TRANSCRIPTIONAL REGULATION OF THE PPAR SIGNALLING PATHWAY BETWEEN PA-AND PAMN-TREATED CELLS................................. 132

FIGURE 5.1: REGULATION OF INTRACELLULAR TAG SPECIES WITH PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. ............................ 155

FIGURE 5.2: REGULATION OF INTRACELLULAR DAG SPECIES WITH PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. ............................ 157

FIGURE 5.3: REGULATION OF INTRACELLULAR CHOLESTEROL AND CHOLESTEROL ESTER LEVELS IN PA- AND PAMN-TREATED FAO HEPATOMA CELLS. 159

FIGURE 5.4: PROFILE OF EXCRETED CHOLESTEROL FOLLOWING PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. ............................ 161

FIGURE 5.5: REGULATION OF INTRACELLULAR AND EXCRETED TOTAL BILE ACIDS IN PA- AND PAMN-TREATED FAO HEPATOMA CELLS. ...................... 163

FIGURE 5.6: REGULATION OF INTRACELLULAR CERAMIDE SPECIES WITH PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. .......................... 165

FIGURE 5.7: REGULATION OF INTRACELLULAR SPHINGOMYELIN SPECIES WITH PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. ................. 167

FIGURE 5.8: REGULATION OF INTRACELLULAR PI SPECIES WITH PA- AND PAMN-TREATMENT IN FAO HEPATOMA CELLS. ............................... 170
FIGURE 5.9: REGULATION OF LIPID SPECIES WITH PA SIDE CHAINS IN PA- AND PAMN-TREATED FAO HEPATOMA CELLS.......................................................... 175

FIGURE 5.10: REGULATION OF THE GLUCONEOGENETIC GENES PEPCK AND G6PASE WITH PA AND PAMN-TREATMENT......................................................... 177

FIGURE 6.1: PROPOSED REGULATION OF LIPID METABOLISM AND GLUCOSE PRODUCTION WITH PA- AND PAMN-TREATMENT............................................ 196

FIGURE A1.1: SCHEMATIC REPRESENTATION OF THE 48 H TREATMENT REGIME FOR THE INDUCTION AND REVERSAL OF PA-INDUCED DYSREGULATION OF INSULIN SENSITIVITY. ................................................................. 204

FIGURE A1.2: INHIBITION OF GLYCOGEN SYNTHESIS BY PA IN C2C12 MYOTUBES. .................................................................................................................. 206

FIGURE A1.3: INHIBITION OF GLUCOSE UPTAKE BY PA-TREATMENT IN L6 MYOTUBES................................................................................................. 210

FIGURE A1.4: PA-INDUCED INHIBITION OF HA-GLUT4 TRANSLOCATION TO THE PLASMA MEMBRANE IN STABLE INFECTED HA-GLUT4-L6 CELLS............... 212

FIGURE A2.1: COMBINATION REVERSAL OF PA-INDUCED GLUCOSE DYSREGULATION USING 0.1 MM METFORMIN AND 2 – 3 MM SODIUM SALICYLATE IN FAO HEPATOMA CELLS................................................................. 217

FIGURE A2.2: COMBINATION REVERSAL OF PA-INDUCED GLUCOSE DYSREGULATION USING 0.25 MM METFORMIN AND 1 – 5 MM SODIUM SALICYLATE IN FAO HEPATOMA CELLS. ................................................................. 219

FIGURE A2.3: COMBINATION REVERSAL OF PA-INDUCED GLUCOSE DYSREGULATION USING 0.5 MM METFORMIN AND 3 – 5 MM SODIUM SALICYLATE IN FAO HEPATOMA CELLS................................................................. 221

FIGURE A4.1: CELL CYCLE PATHWAY. ........................................................................... 223

FIGURE A4.2: OOCYTE MEIOSIS.................................................................................... 224

FIGURE A4.3: TOLL-LIKE RECEPTOR SIGNALLING PATHWAY...................................... 225

FIGURE A4.4: RENAL CELL CARCINOMA................................................................. 225

FIGURE A4.5: LEUKOCYTE TRANSENDOTHELIAL MIGRATION. .............................. 226
FIGURE A5.15: LINOLEIC ACID METABOLISM ................................................................. 243
FIGURE A5.16: NICOTINATE AND NICOTINAMIDE METABOLISM ................................. 243
FIGURE A5.17: VALINE, LEUCINE AND ISOLEUCINE DEGRADATION . ....................... 244
FIGURE A5.18: RIG-1-LIKE RECEPTOR SIGNALLING PATHWAY ...................................... 245
FIGURE A5.19: PENTOSE AND GLUCURONATE INTERCONVERSIONS. ......................... 246
FIGURE A5.20: PRIMARY BILE ACID SYNTHESIS ........................................................... 246
FIGURE A6.1: IDENTIFICATION OF THE SIDE CHAINS OF PI38:5 IN HUMAN PLASMA AS PROOF OF PRINCIPLE ........................................................................................................ 247
FIGURE A6.2: IDENTIFICATION OF THE SIDE CHAINS OF PI38:5 IN VEHICLE-TREATED CELLS ........................................................................................................................................ 248
FIGURE A6.3: IDENTIFICATION OF THE SIDE CHAINS OF PI38:5 IN PA-TREATED CELLS ........................................................................................................................................ 249
FIGURE A6.4: IDENTIFICATION OF THE SIDE CHAINS OF PI38:5 IN PAMN-TREATED CELLS ........................................................................................................................................ 250
List of Tables

TABLE 1.1: WHO GUIDELINES FOR THE DIAGNOSIS OF DIABETES AND OTHER HYPERGLYCAEMIC STATES. .................................................................................................................. 5

TABLE 1.2: PROPOSED SUBTYPES OF INSULIN RESISTANCE AND THE INSULTS THAT CAN LEAD TO THEIR GENESIS IN CELL MODELS. ................................................................. 11

TABLE 2.1: EFFECTS OF ANTI-DIABETIC AGENTS ON GLUCOSE PRODUCTION, CELLULAR VIABILITY (MTT) AND CELLULAR CYTOTOXICITY (LDH RELEASE) IN PA TREATED FAO CELLS. ........................................................................................................... 65

TABLE 2.3: REVERSAL OF THE PA-INDUCED INCREASE IN BASAL GLUCOSE PRODUCTION USING CO-TREATMENT WITH METFORMIN AND SODIUM SALICYLATE. ............................................................... 73

TABLE 3.1: PRIMER SEQUENCES FOR RT-PCR ANALYSIS OF THE SEVEN TARGET GENES IDENTIFIED THROUGH DLDA. ........................................................................................................... 94

TABLE 3.2: GENES IDENTIFIED VIA DLDA AS DISCRIMINATING BETWEEN PA- AND PAMN-TREATED CELLS. ................................................................................................................. 97

TABLE 3.3: CORRELATIONS BETWEEN ANXA3 GENE EXPRESSION LEVELS AND PHENOTYPES LINKED WITH THE METABOLIC SYNDROME IN THE SAHFS (N = 1240). ........................................................................................................... 104

TABLE 4.1: PATHWAYS IDENTIFIED AS SIGNIFICANTLY OVERREPRESENTED IN THE LIST OF DIFFERENTIALLY EXPRESSED GENES BETWEEN VEHICLE- AND PA-TREATED CELLS ........................................................................................................... 116

TABLE 4.2: PATHWAYS IDENTIFIED AS SIGNIFICANTLY OVERREPRESENTED IN THE LIST OF DIFFERENTIALLY EXPRESSED GENES BETWEEN PA- AND PAMN-TREATED CELLS ........................................................................................................... 121

TABLE 5.1: PRIMER SEQUENCES FOR RT-PCR EXPERIMENTS. .................................. 151

TABLE 5.2: EXPECTED FRAGMENTS FROM THE PRODUCT ION SCANS OF PI 38:5. 171

TABLE 5.3: IDENTIFIED PEAKS FROM THE PRODUCT ION SCANS OF PI 38:5. ….. 173
TABLE A4.1: GENES IDENTIFIED VIA DLDA AND RELEVANT 60MER SEQUENCES FROM AGILENT WHOLE RAT GENOME (4X44K) Oligo Microarray Slides (G4131F). .............................................................. 222
## Abbreviation List

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-DOG</td>
<td>2-deoxyglucose</td>
</tr>
<tr>
<td>AS160</td>
<td>AKT substrate of 160 kd</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualisation and Integrated Discovery</td>
</tr>
<tr>
<td>dH2O</td>
<td>Deionised H2O</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet induced obese</td>
</tr>
<tr>
<td>DLDA</td>
<td>Diagonal Linear Discriminate Analysis</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium (cell culture medium)</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FPG</td>
<td>Fasting plasma glucose</td>
</tr>
<tr>
<td>Fxr</td>
<td>Farnesoid X receptor</td>
</tr>
<tr>
<td>G6Pase</td>
<td>Glucose-6-phosphatase</td>
</tr>
<tr>
<td>GSEA</td>
<td>Gene set enrichment analysis</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase 3</td>
</tr>
<tr>
<td>HS</td>
<td>Horse serum</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Insulin receptor substrate 1</td>
</tr>
<tr>
<td>IRS-2</td>
<td>Insulin receptor substrate 2</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleate (C18:2)</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NGT</td>
<td>Normal glucose tolerance</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>NFW</td>
<td>Nuclease free water</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitate (C16:0)</td>
</tr>
<tr>
<td>PAMN</td>
<td>Palmitate, metformin and sodium salicylate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PI(38:5)</td>
<td>Phosphatidylinositol species (38 carbon side chains, 5 double bonds)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PKCɛ</td>
<td>Protein Kinase C-epsilon</td>
</tr>
<tr>
<td>PKCθ</td>
<td>Protein Kinase C-theta</td>
</tr>
<tr>
<td>PKζ</td>
<td>Protein Kinase C-zeta</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute (cell culture medium)</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TAG</td>
<td>Triacylglycerol</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinediones</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1. Complexity of Diabetes Mellitus

Diabetes is a complex and heterogeneous disease. Combinations of varying causative factors, as well as interactions between environmental and genetic factors all play a role in the onset of the disease. This complexity has hindered the development of long-term effective treatment options for patients, and makes understanding the onset of the disease difficult. This thesis focuses on using whole genome microarray technology to study type 2 diabetes (T2D) with unbiased approaches in order to develop individualised therapeutics for individuals with diabetes.

1.1.1. Prevalence and cost of the worldwide diabetes burden

There are almost one million Australians currently diagnosed with diabetes, however it has been estimated that the total number of Australians suffering from diabetes may be closer to 1.7 million due to chronic levels of patients who remain undiagnosed [1]. A further 275 individuals develop diabetes every day in Australia, and the total number of diabetic and pre-diabetic (as determined by fasting blood glucose or HbA$_{1c}$ levels) Australians may number up to 3.2 million [2]. The annual health system costs associated with T2D in Australia has recently been reported as $1.07 billion [3], however the total economic burden of T2D in Australia has been estimated to be as high as $10.3 billion [4]. In the Unites States of America, there are currently 25.8 million people living with diabetes, and this accounts for 8.3% of
the population [5]. This alarming figure is growing rapidly, with 1.9 million people being newly diagnosed in 2010 alone [5]. It has been estimated that there are currently 79 million adults in the US who are pre-diabetic [5]. The costs associated with managing the diabetes epidemic in America were recently estimated at $174 billion annually, and this figure will increase in the coming years as the prevalence of diabetes increases [5]. The projected increase in the prevalence of diabetes, coupled with the already significant economic costs associated with the disease; make the development of alternative effective treatments an urgent priority.

1.2. Diagnosis and Treatment of Type 2 Diabetes (T2D)

1.2.1. Metabolic staging of T2D

The development of T2D is a progressive condition. The onset of insulin resistance can occur in many cases before a clinical diagnosis has been made [6]. The development of insulin resistance leads to hyperinsulinemia, when insulin resistant tissues require higher levels of circulating insulin to maintain glucose homeostasis. This pre-diabetic state is termed impaired glucose tolerance (IGT), in which normoglycaemia is observed along with insulin resistance and hyperinsulinemia.
FIGURE 1.1: Metabolic staging of T2D.
Progression of T2D from early insulin resistance through to beta cell failure and the development of frank T2D. Adapted from [7].

IGT develops to overt T2D when the production of insulin by the pancreas is no longer sufficient to counteract the rising insulin resistance in the peripheral tissues. As blood glucose levels rise, so does the strain on pancreatic β-cells to produce insulin increase. The onset of chronic hyperglycaemia eventually exhausts β-cells and leads to deficient insulin production. When the pancreas is no longer able to cope with the increasing demands of rising blood glucose, overt T2D results (Fig. 1.1).
1.2.2. Diagnosis of T2D

Diagnosis of T2D, and its precursor insulin resistance, is made difficult by the lack of symptoms early in the development of the disease, and it has been estimated that up to half of all cases are undiagnosed [1]. Predictors of risk for the development of T2D and cardiovascular disease include body mass index (BMI), ethnic origin, blood pressure and cholesterol levels [8]. Current clinical guidelines for the diagnosis of diabetes however are based upon blood glucose measures. The World Health Organisation (WHO) standard criteria for diagnosis of T2D involve fasting plasma glucose (FPG) and the response to an oral glucose tolerance test (OGTT). FPG is a measure of plasma glucose after 8 hours of fasting, while the OGTT measures plasma glucose 2 hours following an intake of 75 g glucose. The current guidelines are outlined in Table 1.1. IGT is characterised by peripheral insulin resistance, while defects in insulin secretion coupled with increased hepatic glucose output characterise impaired fasting glucose (IFG) [9]. IGT and IFG are individually strong indicators of risk for the development of T2D, with individuals suffering from both conditions placed at even higher risk [8].
TABLE 1.1: WHO guidelines for the diagnosis of diabetes and other hyperglycaemic states.

<table>
<thead>
<tr>
<th></th>
<th>FPG</th>
<th>OGTT</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT</td>
<td>&lt; 6.1 mmol/L</td>
<td>&lt; 7.8 mmol/L</td>
</tr>
<tr>
<td>IGT</td>
<td>&lt; 7.0 mmol/L</td>
<td>7.8 – 11.1 mmol/L</td>
</tr>
<tr>
<td>IFG</td>
<td>6.1 – 6.9 mmol/L</td>
<td>&lt; 7.8 mmol/L</td>
</tr>
<tr>
<td>Diabetes</td>
<td>≥ 7.0 mmol/L</td>
<td>≥ 11.1 mmol/L</td>
</tr>
</tbody>
</table>

NGT – normal glucose tolerance, IGT – impaired glucose tolerance, IFG – impaired fasting glucose [10].

1.2.3. Current treatments for individuals with T2D

The development of both insulin resistance and impaired glucose tolerance, conditions that precede the onset of T2D, are closely linked with obesity [11]. Excess visceral fat, and the hormones and inflammatory factors it releases, coupled with excess free fatty acid release have been implicated in the development of T2D [12]. For obese patients exhibiting these symptoms, changes to healthier eating patterns and increased exercise can result in improvements to glucose tolerance. However this approach often fails within the first year of treatment, and therefore the use of multiple medications is often required [13]. Lifestyle changes immediately following the diagnosis of T2D can be successful in the early treatment of the disease [14]. The benefit of weight loss on improving glycaemic profile has been convincingly observed in follow up studies of patients with T2D who have had bariatric surgery, with mean sustained weight loss after 10 years >20 kg normalising plasma glucose, HbA1c and plasma insulin levels [15, 16]. However, a lack of diagnosis during the early stages of disease progression, coupled with difficulties in
maintaining non-surgical lifestyle modifications, means that lifestyle interventions are not a treatment option that has been effective in the long term for the majority of patients [13].

Metformin is an oral antidiabetic agent, based upon the molecule biguanide. The primary mechanism of action of metformin involves inhibition of hepatic gluconeogenesis, leading to a reduction in blood glucose levels [17], and a subsequent decrease in blood insulin levels. Metformin has a number of side effects including gastrointestinal symptoms and has been linked with rare cases of lactic acidosis which can be fatal, although evidence for this has been contradicted in some studies [18]. Metformin is one of only two oral anti-diabetic agents on the WHO list of essential medicines. The second oral anti-diabetic to be listed by WHO is the drug family known as the sulfonylureas, the most commonly used drug of which is glibenclamide. The sulfonylureas’ mechanism of action involves inhibition of the pancreatic K-ATP channel sulfonylurea receptor (SUR1/Kir6.2) [19], leading to a depolarisation of the plasma membrane and opening voltage gated Ca2+ channels, which cause increased fusion of insulin-containing granules to the plasma membrane, thereby, increasing insulin secretion [20]. For this reason, the sulfonylureas show their best efficacy in the early stages of the disease when β-cell function is still viable. Side effects associated with the sulfonylureas include hypoglycaemia due to their long half-life in plasma, and weight gain.

The glinides are a family of drugs with a mechanism of action similar to the sulfonylureas, in that they bind to and inhibit the same K-ATP channel (SUR1/Kir6.2) – although at an alternate binding site – to induce insulin secretion from the β-cells
of the pancreas [19]. The glinides have a shorter half-life in blood plasma than the sulfonylureas. As such, some glinides pose a lower risk of hypoglycaemia than some sulfonylureas [21].

Thiazolidinediones (TZDs or glitazones) are an insulin sensitising family of compounds. TZDs are ligands for the nuclear transcription factor peroxisome proliferator-activated receptor γ (PPARγ), as well as weak activators of other PPAR isoforms. It is through activation of the transcriptional regulatory PPAR family that this family of compounds increase the sensitivity of muscle, liver and adipose tissue to the effects of insulin [22]. However, this family of drugs has been linked to serious long-term side effects. Troglitazone, first approved for use in T2D patients in 1997, was withdrawn from the market in 2000 after it was linked to a number of cases of liver dysfunction and failure [23]. Pioglitazone has been linked with increased risk of bladder cancers in adults with T2D [24]. The widely used alternative rosiglitazone has in recent years been linked to increased cardiovascular disease [25]. The drug has been withdrawn from sale in the UK and New Zealand. While still available in the US, rosiglitazone is currently branded with additional safety warnings and restrictions on its use, and sales in recent years have fallen significantly [26].

Glucagon-like peptide 1 agonists (GLP-1 agonists) are mimics of a protein secreted by the L-cells of the small intestine, and act on GLP-1 receptors in pancreatic β-cells to induce insulin release. GLP-1 agonists have also been shown to stimulate β-cell proliferation [27, 28] and suppress glucagon release and gastric motility, while
inducing weight loss. Side effects of GLP-1 agonists include a decrease in gastric motility, responsible for the nausea commonly experienced by patients [29].

Amylin is a β-cell hormone co-secreted with insulin. Amylin lowers blood glucose levels by inhibiting glucagon secretion following a meal, and induces satiety by acting upon the area postrema (AP) neurons within the brain stem [30]. While amylin forms aggregates, which make it unsuitable as a therapeutic agent, amylin agonists such as pramlintide can effectively simulate the effects of the physiological amylin. Like GLP-1 agonists, amylin agonists can induce nausea in patients [31].

Exogenous insulin is a key therapeutic agent for the treatment of diabetes, capable of increasing blood insulin levels when β-cell function has been impaired, and can be given in increasing amounts – alongside other insulin sensitising agents – to overcome insulin resistance [32]. However, insulin is also associated with increases in weight gain, as well as risk of hypoglycaemia if monitoring of blood glucose levels is not rigorously performed.

1.2.4. Problems and adverse effects of current drug therapies

As highlighted above, the currently used range of anti-diabetic medicines have a number of adverse side effects, including hypoglycaemia, fluid retention and weight gain, and gastro-intestinal symptoms. As T2D generally progresses over time to a worsening in glycaemic control, the need to utilise multiple therapies together is unfortunately the reality for many patients with T2D [13]. Difficulties in managing T2D are exacerbated by the fact that the various drugs available have a wide range
of effects in individual patients, in terms of the magnitude of both efficacy and side
effects [32]. In addition to these factors, many of the current drugs used to treat T2D lose their efficacy over time [32]. Therefore, the focus of new treatments has progressed towards personally tailoring pharmacotherapy to suit each patient’s individual characteristics.

We therefore propose that the reason why current therapies do not offer a comprehensive treatment for T2D is that they do not address the underlying heterogeneous nature of the disease. A number of different subtypes of insulin resistance have been described, across a range of different tissues and due to varying insults (see Section 3.1 below). If effective treatments for T2D are to be developed, there is a need to gain a better understanding of the different subtypes of insulin resistance.

1.3. Complexity of T2D and insulin resistance subtypes

1.3.1. Insulin resistance subtypes

Multiple clinical phenotypes such as abdominal obesity [33], polycystic ovary syndrome (PCOS) [34], Cushing’s syndrome [35], lipodystrophy [36], chronic levels of insulin [37], acromegaly (elevated growth hormone) [38], glucocorticoid treatment (eg. during acute lymphoblastic leukemia) [39] and chronic infection [40] are all associated with insulin resistance. The links between the causes of insulin resistance associated with these phenotypes is not obvious since multiple pathways have been implicated in the development of insulin resistance, such as
hyperlipidemia [41], elevated levels of pro-inflammatory cytokines [42] and/or induction of oxidative [43] or endoplasmic reticulum (ER) [44] stress pathways which may be activated individually or concurrently [45]. Superimposed on this network of interactions is the genetic variability of each individual that confers a differential susceptibility to each insult, adding another layer of complexity [46]. However, all of these insults can cause insulin resistance individually or in concert via a number of different mechanisms (Fig. 1.2).

**FIGURE 1.2: The multifactorial nature of T2D.**
A patient’s own genetic susceptibility (I-IV), coupled with multiple insults (A-F) can lead to insulin resistance via multiple mechanisms. Insulin resistance can therefore lead onto frank T2D via numerous routes (from NHMRC grant #533824, Molero, Konstantopoulos, Jowett and Segal).

Insulin resistance is a major risk factor for the development of T2D [47]. Combating insulin resistance provides a key to developing effective treatments for T2D. The aetiology of T2D is multifactorial, with both genetic and environmental factors
involved [48]. Likewise, the onset of insulin resistance is multifactorial and can occur in different tissues and arise from multiple causes as depicted in Fig. 1.2. There are numerous known insults to insulin signalling and action. Insults to insulin action can be both endogenous, such as inflammatory cytokines released in response to a fatty meal, and exogenous, such as the fatty acids themselves, which can lead to the development of insulin resistance. These subtypes can be mimicked in cell culture based models, as shown in Table 1.2.

**TABLE 1.2: Proposed subtypes of insulin resistance and the insults that can lead to their genesis in cell models.**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Causative agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>Cytokines: eg. IL-6, TNFα [42]</td>
</tr>
<tr>
<td>ER Stress</td>
<td>Tunicamycin, Thapsigargin [44]</td>
</tr>
<tr>
<td>Glucocorticoid</td>
<td>Dexamethasone [49]</td>
</tr>
<tr>
<td>Hyperinsulinemia</td>
<td>Chronic elevated insulin levels [50]</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>ROS: eg. Superoxide anions [43]</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>Long chain, saturated FFAs: eg. Palmitate (PA; 16:0) [41]</td>
</tr>
</tbody>
</table>

While there are a number of factors, which may lead to the development of insulin resistance in various tissues, they do not necessarily develop in complete isolation, and signalling crosstalk between the various models mentioned above occurs. For example, hyperlipidemia-induced insulin resistance has also been linked to increased generation of the inflammatory cytokine TNFα through activation of the pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [51, 52].
We therefore propose that treating the underlying subtype(s) of insulin resistance in a patient with either early insulin resistance or frank T2D will have increased efficacy for the long-term maintenance of euglycaemia. We aim to identify a gene expression signature (GES) based on different known causes of insulin resistance. The generation of a GES for the profiling of patients would then allow the stratification of diabetic patients according to the underlying insulin resistant condition(s). We therefore hypothesise that we can use the GES profiles to identify the subtype(s) contributing to a patient’s insulin resistance. This would allow for tailoring of a patient’s medication targeting their insulin resistance subtypes for personalised management of their metabolic dysregulation. This will be discussed in further detail below.

1.3.2. The rising worldwide prevalence of obesity

Obesity is a major risk factor for the development of insulin resistance and T2D [53, 54], and the relationship between weight gain and decreased insulin sensitivity has been established for many years [55]. Obesity is widespread in the western world, with recent WHO data estimating 64% of Australians are either overweight or obese, with 27% being obese [56]. If weight gain in Australian adults continues at the current projected rate, by 2025, 80% of the population will be overweight or obese, along with 30% of children [57]. The recent US National Health and Nutrition Examination Survey (NHANES) estimated that for Americans aged 20 and above, 67% are either overweight or obese, while 34% were obese [58]. The WHO estimates that in 2005 there were 1.6 billion adults worldwide who were overweight, at least 400 million of which were obese. These numbers are projected
to increase to 2.3 billion overweight and at least 700 million obese adults by 2015 [59]. The increasing epidemic of obesity will further increase the prevalence of insulin resistance and T2D within society, making the development of effective treatments a critical challenge for the 21st century.

1.4. Obesity

1.4.1. Free fatty acids

As one of the primary risk factors for the development of T2D, obesity warrants extensive study as a target for the development of additional and alternative therapies. The defining characteristic of obesity is increased adiposity. Increased availability of free fatty acids (FFA) in patients with obesity plays a critical role in the development of insulin resistance [60]. There are numerous factors in obesity which can lead to increases in circulating FFA levels, including exceeding the storage capacity of adipose tissue by excess caloric intake [61], and adipose tissue stimulation by the paracrine cytokine TNFα which induces triglyceride metabolism and FFA release [62]. Insulin resistance in adipose tissue can also lead to excess FFA release, due to suppression of the antilipolytic effects of insulin [62]. The direct effects of increased circulating FFAs on macrophages to stimulate release of pro-inflammatory cytokines such as TNFα and IL-6 has also been recently described (for a review see [63]). The onset of insulin resistance caused by FFA is therefore highly complex, and although direct action upon target tissues have been described, there are secondary actions upon other tissue types that further complicate the pathology of the disease. For example, following both PA-treatment of mouse
C2C12 myotubes and lipid infusion (Liposin II) in individuals has been shown to induce intramuscular generation of the inflammatory cytokine TNFα through activation of the pro-inflammatory transcription factor NF-κB [51, 52], which in turn induce inflammatory-based insulin resistance in other peripheral tissues, as has been shown in 3T3-L1 adipocytes and FAO hepatoma cells [42]. Given the increasing prevalence of obesity around the world, dissecting the mechanisms by which FFAs contribute to insulin resistance may identify new avenues for effective treatment regimes.

1.4.2. Effect of dietary free fatty acid composition on insulin signalling

The effects of FFAs on insulin sensitivity both in vivo and in vitro have been studied extensively in recent times. The KANWU study (named for the locations of data collection centre: Kuopio, Aarhus, Naples, Wollongong and Uppsala) remains one of the largest controlled studies looking into the effects of fat composition in the diet on insulin sensitivity. Healthy individuals (n = 162) were placed on one of two isoenergetic diets for 90 days: either high in monounsaturated (MUFA diet, high in oleic acid, n = 79) or saturated (SAFA diet, n = 83) fatty acids. Individuals on the SAFA diet were found to have reduced insulin sensitivity index compared to baseline levels (-10%, p=0.03), while no change was observed in the MUFA diet [64]. The study also found that only for those subjects with total fat intake below the median (37% of energy), there was a positive effect for substituting saturated fatty acids with mono-unsaturated fatty acids; individuals on the MUFA diet were found to have an increased insulin sensitivity index (+8.8%, p=0.03) [64]. The KANWU
study was one of the first human studies to link both the type and quantity of fatty acid in a dietary intake to decreases in insulin sensitivity. The relationship between dietary fatty acid composition has been extensively studied (and reviewed [65-68]), and it has been consistently observed that diets enriched in saturated fatty acids are inversely correlated with insulin sensitivity in individuals [69-73].
1.4.3. Normal metabolic interplay between carbohydrate and fatty acid metabolism/oxidation

**FIGURE 1.3: Synthesis and oxidation of intracellular fatty acids.**
Intracellular fatty acids derived from de novo synthesis or uptake from the plasma is oxidised in the mitochondria. Synthesis and oxidation of fatty acids is metabolically regulated by the availability of carbohydrate as an energy source, mainly through the activation/inhibition of acetyl-CoA carboxylase (ACC). Figure adapted from [74].
While availability of glucose can modulate the relative rates of fatty acid synthesis and oxidation through activation/inhibition of ACC and subsequent malonyl-CoA production (Fig. 1.3), in states of excess lipid availability, impaired glucose metabolism can arise. There have been numerous studies into the mechanism of fatty acid-induced insulin resistance. Although the mechanisms are not completely understood, there is currently a vast body of evidence pointing to a number of mechanisms by which FFAs induce insulin resistance in key insulin responsive tissues such as skeletal muscle and liver, and this will be summarised below.

1.4.4. Effects of saturated free fatty acids on insulin sensitivity in skeletal muscle

Increased circulating FFA levels have been shown to cause dose-dependent decreases in glucose uptake, a reduction in glycogen synthesis and a reduction in carbohydrate oxidation in muscle biopsies of healthy individuals [75]. *In vitro* cell culture studies have shown a consistent link between long chain saturated free fatty acids such as PA (16:0), stearate (18:0), arachidate (20:0) and lignocerate (24:0) and the onset of cellular insulin resistance in muscle cells such as mouse C2C12 myotubes [41]. Shorter chain FFAs such as laurate (12:0) and myristate (14:0), as well as unsaturated fatty acids such as oleate (18:1) have not been shown to have this inhibitory effect on insulin signalling [41]. *In vivo* studies in mice using muscle-specific over expression of lipoprotein lipase (LPL) have shown that by directly increasing lipid metabolites such as fatty acyl-CoAs and ceramides, muscle insulin signalling and action, as well as glucose tolerance was decreased [76].
1.4.4.1. Ceramide accumulation and the atypical protein kinase C-zeta (PKCζ)

Isolated mouse soleus muscle cells incubated with the saturated fatty acid PA (1 mM for 6 h) have increased intracellular levels of ceramide [77]. Ceramide, a key cellular metabolite of PA, plays a key role in the development of insulin resistance in skeletal muscle. PA is converted within cells to the intermediate palmitoyl-CoA. This intermediate is then utilised within the cell for the synthesis of ceramide and diacylglycerol. Ceramide is synthesised from the precursor molecule palmitoyl-CoA and serine, with the rate-limiting step to this reaction being the availability of these two molecules [61, 78].

Ceramide has been shown to down-regulate insulin signalling by inhibiting the kinase activity of the key insulin signalling intermediate AKT [61, 79, 80]. Ceramides localise within lipid rafts of plasma membranes, leading to phosphorylation of PKCζ at the plasma membrane. Increased activation of PKCζ leads to Ser34 phosphorylation of AKT, inhibiting the kinase activity of AKT [81]. Inactivation of AKT decreases phosphorylation of glycogen synthase kinase 3 (GSK-3; leading to activation of GSK-3), which can then inhibit glycogen synthase activity, thus leading to an inhibition of glycogen synthesis from glucose. AKT inhibition also leads to a decrease in translocation of the insulin responsive glucose transporter GLUT4 to the cell membrane, due to reduction in downstream signalling of intermediates such as AKT substrate of 160 kd (AS160). As a result of inhibited AKT signalling, there is decreased glucose uptake and utilisation by the cell in response to insulin. Such effects upon insulin sensitivity have also been demonstrated in vivo. Diet-induced obese (DIO) mice exhibit a significant increase in their gastrocnemius ceramide
content. Upon treatment with myriocin, a specific inhibitor of *de novo* ceramide synthesis, the increase in gastrocnemius ceramide content was reversed (141.5 ± 15.8 nmol/g dry weight in DIO controls vs. 94.6 ± 10.2 nmol/g dry weight in the myriocin treated DIO mice), which correlated with increased insulin sensitivity and glucose tolerance (\(R=0.61, p<0.05\)) as measured by GTT [82].

### 1.4.4.2. Diacylglycerol (DAG) accumulation and Protein Kinase C activation

DAGs have also been implicated in the development of insulin resistance in muscle cells. DAG has been shown in C2C12 myotubes to accumulate following 16 h incubation with saturated fatty acids such as PA (0.5 mM) [83]. DAG accumulation in C2C12 cells has been shown to inhibit upstream mediators of AKT signalling [41] through binding to the regulatory domain of novel PKC isoforms, thus activating their inhibitory signalling properties. Activation of PKC\(\varepsilon\) by DAGs synthesised from PA leads to increased Ser/Thr phosphorylation of the insulin receptor (IR), decreasing its tyrosine kinase activity, and thus inducing insulin resistance by down regulating IR-downstream signalling (reviewed in [84]). Activation of PKC\(\theta\) by DAG species also mediates the inhibitory effects of DAG accumulation, phosphorylating the insulin receptor substrate 1 (IRS-1) and decreasing downstream signalling in C2C12 myotubes [85]. In addition, activated PKC\(\theta\) has also been implicated in the induction of NF-\(\kappa\)B signalling [86], leading to pro-inflammatory cytokine production – another mediator of insulin resistance in muscle, adipose and the liver.
1.4.4.3. **Reactive oxygen species (ROS) accumulation and c-Jun NH$_2$-terminal kinase (JNK) activation**

FFAs also inhibit insulin signalling in skeletal muscle by generation of reactive oxygen species (ROS). Oxidation of PA in isolated primary rat skeletal muscle cells has been shown to increase production of the radical superoxide, through $\beta$-oxidation in the electron transport chain, and also through the effects of NADPH oxidase [87, 88]. ROS can activate the stress kinase (JNK), which phosphorylates IRS-1 on Ser307, inhibiting its association with IR and increasing IRS-1 protein degradation [89], thus impairing downstream signal transduction.
1.4.5. Effects of free fatty acids on insulin sensitivity in the liver

**FIGURE 1.4: Interplay between hepatic fatty acid utilisation and glucose production.**

Intracellular fatty acids are oxidised for energy, resulting in acetyl-CoA production. Acetyl-CoA feeds into production of ketone bodies (energy source) or via the citric acid cycle into hepatic glucose production. Figure adapted from [74].

In normal metabolic states, lipid availability in the liver provides an energy source directly to hepatocytes, as well as systemically via production of ketone bodies and glucose production (Fig. 1.4). When lipid-oversupply arises, these metabolic states are dysregulated, resulting in the induction of hepatic insulin resistance and increased glucose production, a key feature of T2D.
The general effects of saturated fatty acid oversupply on hepatic insulin sensitivity have previously been shown. PA-treatment (0.25 mM, 12 h) of FAO hepatoma cells decreased insulin-stimulated IR and IRS-2 tyrosine phosphorylation, reduced phosphatidylinositol 3-kinase (PI3K) activity and a reduction in AKT Ser473 phosphorylation [90]. As the primary site of gluconeogenesis within the body, the onset of insulin resistance in the liver leads to increased levels of hepatic glucose production [91]. The liver is therefore a primary target in the development of new therapies against insulin resistance and T2D.

1.4.5.1. **DAG accumulation and Protein Kinase C-epsilon (PKCε)**

DAGs and associated PKCε activation play a key role in regulating insulin sensitivity in the liver (reviewed in [92]). When incubated with recombinant active IR protein, recombinant active PKCε dose-dependently inhibited IR kinase activity [93]. Treatment with antisense oligonucleotides towards PKCε in rats decreased hepatic PKCε gene expression and protein levels, and lowered fasting plasma insulin while maintaining fasting plasma glucose levels [93]. Treatment with PKCε antisense oligonucleotide in 3 day high-fat fed rats restored impaired IR kinase activity [93]. Compared with wild-type littermates, *PKCε*−/− mice did not develop glucose intolerance following 1 week of high-fat feeding, and furthermore showed increased hepatic lipid accumulation [94]. Increased accumulation of DAGs, triacylglycerols (TAGs) and cholesterol in high-fat fed *PKCε*−/− mice compared with high-fat fed wild-type littermates was associated with increased fatty acid
esterification and altered protein levels for regulators of lipid metabolism including carboxylesterase ML1 and acyl carrier protein, in parallel with decreased energy expenditure [94]. This suggests that in the face of short-term lipid overload, PKCε deletion redirects fatty acids away from beta-oxidation towards esterification, which in primary hepatocytes from PKCε−/− mice resulted in decreased production of ROS [94]. These findings implicate PKCε in the induction of lipid-induced hepatic insulin resistance, and suggest a role for the modulation of hepatic fatty acid metabolism in restoring hepatic glucose metabolism.

1.4.5.2. Hepatic ceramide accumulation

Ceramide accumulation has been associated with the development of hepatic insulin resistance, however the evidence supporting this link is controversial. Inhibition of ceramide synthesis via myriocin in DIO mice has been shown to increase basal and insulin-stimulated AKT phosphorylation in the liver, as well as skeletal muscle [95]. It is not clear however whether these effects are due to the prevention of ceramide accumulation in the liver or systemic effects resulting from increased insulin sensitivity in the muscle. While there have been reports of hepatic accumulation of ceramide in obese, insulin-resistant Zucker rats [96], there is some confusion in the literature about the role of ceramide in hepatic insulin resistance. It has been reported that hepatic insulin resistance can occur independently of ceramide accumulation [97], while other groups have reported hepatic ceramide accumulation without development of insulin resistance (assessed by hyperinsulinemic-euglycaemic clamp) in liver-specific acylCoA:diacylglycerol acyltransferase 2 (DGAT2) overexpression in mice [98], although this latter finding
has been refuted by other groups showing hepatic DGAT2 overexpression in mice lead to both hepatic steatosis and insulin resistance [99]. It can be concluded from published studies to date that accumulation of total ceramide may not be necessary for the development of hepatic insulin resistance in vivo. It has recently been reported that the liver has the ability to rapidly excrete newly synthesised ceramide [100], which may explain the contradictory reports of hepatic ceramide accumulation across different models of hepatic insulin resistance.

1.4.5.3. Reactive oxygen species (ROS) accumulation and JNK activation

ROS released by the mitochondria in response to PA β-oxidation also plays a role in the development of insulin resistance in the liver [88]. PA is rapidly routed via carnitine parmitoyltransferase-1 (CPT-1) through β-oxidation in the mitochondria, increasing production of ROS [90, 101]. Inhibiting β-oxidation in FAO hepatoma cells with etomoxir (100 μM, a competitive CPT-1 inhibitor) restored PA-induced (0.25 mM) IR tyrosine phosphorylation defects [90], and in H4IIEC3 hepatoma cells reduced PA-induced (0.25 mM, 8 h) ROS production by 80% [101]. PA-induced ROS production in H4IIEC3 cells was observed in parallel with 2.5-fold activation of JNK (Thr183/Tyr185) phosphorylation [101]. Activation of JNK leads to serine phosphorylation of both IRS-1 and IRS-2 [102, 103], down-regulating signalling as described for JNK activation in skeletal muscle above. Hepatic overexpression of dominant-negative JNK in obese diabetic mice decreased fasting plasma glucose levels and decreased hepatic glucose production, while overexpression of wild-type JNK in normal mice elevated fasting plasma insulin and increased hepatic glucose
production [104]. Therefore, enhanced activation of JNK can induce decreased hepatic insulin action and increased hepatic glucose production both \textit{in vitro} and \textit{in vivo}.

\subsection*{1.4.5.4. Induction of the pro-inflammatory NF-kB pathway}

Lipid-induced activation of the pro-inflammatory NF-kB pathway has been observed \textit{in vivo} in rodent models using lipid-heparin intraperitoneal injection [105]. While the induction of the NF-kB signalling pathway in hepatic lipid-induced insulin resistance has been consistently observed, the exact mechanism by which this occurs is not clear [105]. Transgenic mice lacking hepatic IKK-β retain hepatic insulin sensitivity, despite developing insulin resistance in muscle and fat tissues, suggesting that activation of the NF-kB pathway is dependent upon activation of IKK-β [106]. Induction of IKK-β activity leads to a ubiquitin-mediated degradation of the NF-kB suppressor IkB-α [107]. NF-kB is then free to translocate to the nucleus, where transcription of multiple inflammatory cytokines is induced including TNF-α and IL-6 [105], which are both established mediators of insulin resistance in adipose [108], muscle [109] and liver tissue [110].

\subsection*{1.4.6. Insulin independent effects on basal glucose production}

In addition to the inhibitory effects on hepatic insulin sensitivity, elevated lipids can also increase glucose production \textit{in vivo} independent of insulin sensitivity [111]. One of the main effectors of the lipid-induced driven increase in glucose production is increased formation of acetyl-CoA, which along with increasing substrate availability for hepatic glucose production (Fig. 1.4) also acts as an allosteric
activator of pyruvate carboxylase (enzyme described below). The allosteric activation of pyruvate carboxylase by acetyl-CoA has been extensively studied (reviewed in [112]), and increased hepatic acetyl-CoA levels have been implicated in raising basal glucose production in exercise-induced states since the early 1980s [113].

1.4.6.1. Acetyl-CoA and pyruvate carboxylase

Pyruvate carboxylase is a tetrameric enzyme which catalyses the initial reaction in the gluconeogenesis pathway, regulating the synthesis of oxaloacetate from pyruvate and HCO\textsubscript{3} (reviewed in [114]). Acetyl-CoA, and to a lesser extent fatty acyl-CoAs, bind to and stabilise the tetrameric structure of pyruvate carboxylase. In addition to preventing dissociation of the tetramer [115], acetyl-CoA binding also enhances the coupling of the cleavage of ATP (in the first phase of the synthesis reaction) with pyruvate carboxylation (in the second phase of the synthesis reaction), thereby making the reaction more efficient [116]. Increased flux through pyruvate carboxylase therefore promotes downstream production of glucose in the liver.

1.4.6.2. p38 MAP-kinase activation by fatty acids

Protein levels of p38 mitogen-activated protein kinase (MAPK) are elevated in the livers of \textit{db/db} mice [88]. In primary mice hepatocytes, p38 MAPK is phosphorylated (Thr180/Tyr182) by a 20 min treatment with PA (0.1 – 1.5 mM), linoleate (0.1 – 1.5 mM), oleate (0.5 – 1.5 mM) or capric acid (1 – 1.5 mM) [117]. Oleate-treatment (0.5 mM) for 4 h in primary mice hepatocytes increased glucose production, which was
reversed by co-treatment with 5μM of the p38 MAPK inhibitor SB203580 [117]. The oleate-stimulated increase to glucose production and p38 MAPK occurred in parallel with increased gene expression levels of phosphoenolpyruvate carboxykinase (PEPCK, the rate limiting enzyme in the glucose production pathway). The oleate-induced increase to PEPCK gene expression levels was also inhibited by the p38 MAPK inhibitor SB203580 [117]. The translation of PEPCK by p38 MAPK is dependent upon the transcriptional co-activator peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC1α) [117], which along with FOXO1 regulates PEPCK gene expression levels [118, 119].

1.4.7. Lack of effect of free fatty acids on insulin sensitivity in adipose tissue

Incubation of 3T3-L1 adipocytes with 0.75 mM PA for 16 h did not lead to increased intracellular DAG or ceramide levels, nor an inhibition of AKT (Ser473) phosphorylation or glycogen synthesis [41]. Unlike skeletal muscle or liver cells [120], adipose tissue stores fatty acids mainly as triglycerides [121]. The ability of adipose tissue to preferentially convert fatty acyl-CoAs into triglycerides thus prevents the conversion of fatty acids into harmful metabolites such as DAG and ceramide [41], therefore protecting adipose tissue from fatty acid induced insulin resistance.
1.5. Previous approaches for characterising insulin resistance

1.5.1. Classical single target-based approaches

Classical approaches for dissecting insulin resistance involved targeting signalling defects in both in vitro and in vivo models of insulin resistance. These approaches – utilising western blotting for proteins and PCR for gene expression data – have enhanced our knowledge of insulin resistance and the mechanisms by which insulin signalling is impaired. However such approaches rely upon previous knowledge to build a network of signalling connections, and implicating signalling defects in the observed in vitro or in vivo model. As is becoming increasingly clear, signalling networks within cells are far more complicated than previously thought. Single insults (such as fatty acids) not only impact upon insulin signalling directly, but also on numerous signalling cascades such as inflammatory pathways, which may be either distally related to insulin signalling or not related at all. This will result in activation of many kinases, and modification of transcription of a number of genes, in the process of the cell reaching an equilibrium state.

We now know that the single target or pathway approaches provide too narrow a window to appreciate the changes induced in complex disease states. While the contribution of the single target / pathway approaches cannot be denied, in terms of expanding our knowledge base, a wider approach is now required for the development of the next line of therapies.
1.5.2. Endpoint-based approaches

Endpoint-based approaches have been significant in furthering our understanding of the development of diabetes. Utilising insulin signalling endpoints such as hepatic glucose production or muscle glucose transport can provide a more global overview of the cellular state compared with the phosphorylation of a single kinase amongst a signalling network. The discovery of new therapies targeted against endpoints allows us to bypass the upstream complexity that hinders the target-based approaches.

1.5.3. ‘Omics’ approaches

The development of powerful platform technologies such as microarrays has led to a vast increase in the utilisation of the ‘omics’ studies. Current mass spectroscopy techniques allow for the study of nearly the entire lipid or specific protein fractions within a sample, allowing characterisation of disease states in an unprecedented way. The requirement to investigate and treat many diseases with multifactorial natures has necessitated the need for more powerful, unbiased technologies to give researchers a “global” view of disease states. The search for effective early diagnostic tools, insight into the development of disease states, and the development of new therapies are increasingly relying on one or more of these new platform approaches.

In the context of obesity, lipidomic approaches are proving to be very useful in identifying characteristic changes in tissue-specific lipid profiles of patients with T2D [122], which has been made possible by advances in mass spectroscopy techniques.
Advances have also been applied to the proteomic field. Techniques such as Stable Isotope Labelling by Amino acids in Cell culture (SILAC) are proving to be powerful in furthering our knowledge of insulin signalling cascades in both normal and insulin resistant states, by allowing the investigation of a large number of proteins at once across multiple samples [123].

1.5.4. Genomics-based approaches

Developed in the mid 90’s for the analysis of the expression of multiple genes in parallel [124], microarray technology can now be used to assess the expression of tens of thousands of genes in a sample simultaneously. This provides a powerful tool to assess whole cell transcriptional events for any given cell or tissue in any biological state. Microarray technology has a range of applications including identifying disease-causing genes, identifying targets for new therapies and prediction of drug responsiveness [125].

Two major applications for microarray technology involve: 1) examining gene sets for pathway analysis, and 2) examining differentially expressed genes between two or more experimental conditions [126]. Gene set enrichment analysis (GSEA) involves analysis of a gene list, ranked according to the difference in expression between the phenotypes or treatments being investigated. The goal of GSEA is to determine whether members of specific gene sets (grouped on functional similarity), are ranked together towards the top or bottom of the list. GSEA therefore indicates whether a correlation exists between differential expression of that set of genes, and the specific phenotype being investigated [127, 128]. This
pathway analysis approach to dissecting disease is complimented well by proteomic approaches that can similarly be used for pathway analysis.

The second of the two applications involves performing microarray analysis on gene sets from multiple experimental conditions, and can be used to identify differentially expressed genes in differing disease states. This ‘shotgun’ style approach to genome analysis can yield previously unknown information about the regulation of disease states at the transcriptional level, which can have important implications for understanding the pathophysiology of disease. The set of differentially expressed genes can also be used for a diagnostic approach to the disease. Applying Bayesian Linear statistical modelling to gene sets allows for selection of a relatively small gene set which can characterise the particular biological state of the cell or tissue being investigated [129]. This process statistically evaluates which set of genes have the greatest differential expression between the conditions tested, and identifies a ‘fingerprint’ indicative of the biological state of the cell or tissue involved, known as a gene expression signature (GES). Previously, GESs have been applied to the field of cancer research, for applications such as classifying tumour types and predicting tumour response to chemotherapy. By classifying tumours into distinct types, and with knowledge of how each type will respond to particular therapies, clinicians are therefore able to treat patients more effectively by personalising treatment regimens [130]. Personalised medicine approaches such as this are becoming increasingly important tools in fighting diseases and the use of GESs is likewise increasing in disease research.
1.6. Gene Expression Signatures

1.6.1. Gene expression signatures as a diagnostic tool

First described in 2000, GESs were developed in the field of cancer research. The differences in patient response to therapies led researchers to believe that groups of cancers that could not be histologically characterised were actually a heterogeneous group of tumours. Seeking a non-biased method for classification, gene expression data was investigated to search for patterns that could differentiate classes of B-cell lymphomas with differing patient survival rates [131]. The main outcome of the study was the finding of two subgroups, classified on the basis of differential gene expression of hundreds of genes, with differing survival outcomes for patients. This early study was instrumental in highlighting the use of gene expression data as a disease classification tool. The power of the GES approach is that entire genome datasets are narrowed down to the smallest number of genes capable of robustly characterising differences between biological samples. Using complex statistical analysis of large datasets, the prediction power of these small subsets of genes has been shown to approach equivalence to the whole dataset [132]. Once developed, the GES tool allows for rapid, reliable characterisation of various cellular states, which has a number of important applications.

Accurate classification of disease states plays a vital role in diagnosis and treatment. GESs have been successfully used in a number of different cancer types including breast [133, 134], gastric [135], lung, colon and ovarian cancer [136] to aid in
prediction of survival, and to guide clinicians in choice of treatments for their patients. Recently, GESs have even been applied to predicting the likelihood of side effects in patients treated by acute radiotherapy [137].

Using GES technology for prediction and/or classification however represents only part of the potential of such an approach. The use of GESs for the discovery and development of new therapies is perhaps the most promising application of this technology. The use of GESs to discover new therapies is especially powerful as it can be applied whenever a specific disease endpoint (such as insulin resistance) is known, even if intermediate signalling steps or the molecular targets responsible for the disease state have not yet been identified. Provided a model for the disease of interest can be developed, high throughput screening of small molecule libraries can be performed by assessing the effects of those agents on the mRNA levels of the genes identified as the GES. The GES approach has been used in a number of cancer models to identify new therapies, which have increased efficacy over current treatments. For acute myelogenous leukaemia, the identification of inducers of terminal differentiation has opened up new therapeutic avenues previously unavailable [138]. For the treatment of Ewing sarcomas, the targeting of the EWS/FLI oncoprotein had previously been unsuccessful with screening approaches, until the GES approach was used successfully to identify cytosine arabinoside as a modulator of the EWS/FLI oncoprotein [139].

What makes GESs unique is that the GES genes are not limited to genes known to be involved in the particular physiological process being investigated. A GES is the minimal set of genes that best defines the difference between two biological
samples – be that a disease state or the physiological response to a particular drug or chemical. While it is possible that an individual GES gene may play a role in the specific model being investigated (see section 7.4. below: the protective inflammatory response gene STEAP4 [140] was identified in the TNFα-based GES previously developed by our laboratory [141]), the unbiased nature of gene selection does not necessitate such a relationship, and therefore the role of any GES genes in contributing to the model phenotype must be confirmed with subsequent studies.

1.7. Overview of GES Development

**FIGURE 1.5: Development procedure for a GES for insulin resistance.** mRNA is extracted from *in vitro* cell culture models of insulin resistance, and analysed using whole genome microarrays. The GES genes are identified, and validated in human tissues. The validated GES can then be used to identify novel treatments, as well as stratify patients. This allows for the personalised treatment of T2D. Based upon [138].
We propose that GESs can be applied to dissect and study insulin resistance subtypes. The GES methodology described here can be undertaken in either animal tissues or cell culture models. Due to the high reproducibility required when extracting the data from global platforms such as microarray technology, we have found that working in cell culture systems is the most robust and consistent approach. The modelling of insulin resistance subtypes in the GES models involves the use of a specific insults associated with the induction of insulin resistance in individuals, including saturated fatty acids (PA) or mediators of chronic inflammation (TNFα).

The development of a GES in cell culture requires modelling three distinct cellular states relating to insulin sensitivity. The first state is that of a ‘healthy’, insulin sensitive cell. The second state is that of a ‘diseased’, insulin resistant cell. This is achieved by treatment of the target cells with the insulin resistance insult such as TNFα or PA. The third state represents a ‘recovered from disease’ state, which is achieved by treating insulin resistant cells with a cocktail of anti-diabetic agents to restore insulin action (Fig. 1.6). The definition of these three states is deliberate and critical to the integrity of the GES. Insulin resistance in this model system is measured using a key endpoint of insulin action, such as glucose uptake in muscle or adipose tissue, or glucose production in the liver, and will be discussed in further detail below.
FIGURE 1.6: Distinct cellular states required for development of a GES for insulin resistant subtypes.
The development of a GES for PA-induced insulin resistance requires modelling of three cellular states: “healthy”, “diseased” and “recovered from disease” states – which model the transcriptional responses to the development and recovery from lipid-induced insulin resistance in the liver of a patient with T2D.

1.7.1. Characterising insulin resistance in vitro

In order to effectively model insulin resistance in vitro, an endpoint measure of insulin action is required. Cell-based models offer a number of assays that can be used to determine insulin signalling in both sensitive and insulin resistant states. In vitro models of insulin resistance can be developed in each of the main insulin sensitive tissues; muscle, adipose and liver. One key measure of insulin action in muscle and adipose cells is glucose uptake. In liver cells, regulation of gluconeogenesis by insulin is one of the key endpoints of insulin action. These assays work by measuring the relevant endpoint (glucose uptake or gluconeogenesis) in the presence and absence of an insult to characterise the level
of insulin sensitivity. As the *in vitro* cell culture model must be manipulated from healthy to diseased and then restored, a robust and large dynamic range is needed in the bioassay used to measure the insulin resistance endpoint parameter.

Reversal of insulin resistance involves assessing a wide range of known insulin sensitisers in the model of choice. A combination therapy that is able to fully reverse insulin resistance is selected, based upon its ability to not only reverse insulin resistance, but also avoid negatively impacting upon cellular viability. Combination therapy is required, as this will ensure that the GES is characteristic of an overall insulin resistant state that has been reversed by a multi-target approach. There is a greater chance that in drug development the GES will identify novel therapies, rather than the individual therapies used in its creation – as may happen with a single treatment GES. Potential reversers of insulin resistance include known anti-diabetic drugs such as the biguanide metformin [142], TZDs such as rosiglitazone [143], chemical chaperones such as tauroursodeoxycholic acid (TUDCA) [144], antioxidants such as N-acetylcysteine (NAC) [145], and non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin [42, 146].

### 1.7.2. Identification of putative GES genes

To apply the GES approach to insulin resistance, firstly we will assess the significant changes in gene expression levels between the ‘healthy’ insulin-responsive cells, and the ‘insulin resistant’ cells to identify the genes that change in response to the insulin resistance-inducing insult. In order to determine which genes are being affected due to insulin resistance and not non-specific changes induced by the insult
per se, the changes induced by the ‘recovered from disease’ state will be assessed. Only those genes whose expression levels were significantly changed in the ‘diseased’ state, and then changed again in the reverse direction in the ‘recovered from disease’ state are used for the development of the GES. It is this group of genes whose expression are linked to the insulin resistant state of the cell or tissue.

The identification of candidate genes for GES development has been previously achieved using two complementary methods: (i) Diagonal Linear Discriminate Analysis (DLDA) coupled with signal-to-noise ratio statistic [138, 141], and (ii) Bayesian statistical modelling [141, 147]. Both approaches have been previously used to identify candidate GES genes for a TNFα-based insulin resistance GES [141]. The main advantage of the Bayesian modelling approach is the ability to calculate the β-correlation coefficient for each GES gene, which accounts for the relative contribution of each gene to the predictive power of the GES as a whole, as well as the variance of each GES gene, which allows for the predictive power of the whole GES to be calculated. The Bayesian approach is highly complex and requires powerful computing resources, and it has been previously found that the genes identified via DLDA have also been identified via the Bayesian approach – validating the use of the less complex DLDA approach as an early identifier of candidate GES genes [141].

Once the GES is developed from a cell culture model, the biological relevance of an in vitro-derived GES requires validation in human tissue. Validation of the GES in human cohorts tests for a correlation between phenotypes characteristic of the metabolic syndrome, and similarity to the GES profile for each subject. If it can be
shown that the patients with expression profiles most similar to the GES showed a greater degree of insulin resistance (such as indicated by HOMA-IR score), the GES is considered to be a valid discriminator of insulin resistance in human patients.

1.7.3. Applications of GES in the development of personalised treatment for individuals with T2D

1.7.3.1. Using GES to stratify patients with T2D

The GES holds promise for personalised treatments for patients by allowing the stratification of patients based on subgroups of insulin resistance. Once patients are sub-grouped, treatments can be personalised to their individual diagnosis, leading to improved health outcomes. The sub-grouping of patients according to the GES involves measuring the expression levels of the GES genes in the patient. Regardless of which tissue or cell type the GES is derived from, a non-invasive, easy to obtain sample is needed to facilitate screening of as many individuals as possible. A blood sample is ideal for these requirements. Lymphocyte gene expression profiles have been shown to correlate well with gene expression profiles of insulin responsive tissues including liver and adipose tissue [148]. We propose that by measuring the expression levels of the GES genes in patients’ white blood cells, we can subtype patients according to one or more GES. The GES(s) which best correlates with the gene expression pattern of a patient’s white blood cells will therefore indicate a specific avenue of treatment(s) for that patient (see Fig. 1.7).
Chapter 1: Introduction

1.7.3.2. Using the GES to develop “targeted” therapies

The GES can be used to aid in the development of new therapies for T2D, by allowing for high throughput screening for new drugs with insulin sensitising and anti-diabetic properties. Screening of chemical libraries can be rapidly performed in 96 well-plates, alongside control wells containing the three distinct cellular treatments from which the GES was developed (“healthy”, “diseased” and “recovered from disease”, see Fig. 1.6). After measuring the mRNA levels of the GES genes in the treated cells, this has been previously achieved using a custom
algorithm to transform the relative gene expression levels for each GES gene into a single “GES score”. Firstly, the expression for each gene was expressed relative to the total summed gene expression for the group of GES genes, then standardised (z score) to compare across all genes and samples. The β-correlation coefficient for each GES gene was then multiplied against the standardised gene expression levels and summed to form the “GES score” [141]. The chemicals with a positive “GES score” mimic the GES profile of successful reversal of insulin resistance, while those with a negative “GES score” mimic the GES profile of induced insulin resistance. The drugs with a positive “GES score” were then validated both in vitro and in vivo to assess their anti-diabetic and/or insulin sensitising properties [141, 149]. We propose that new therapies identified via this approach may show increased efficacy in treating patients subtyped by the same GES. The subtyping of patients according to GES, as well as the potential for targeted therapies against each subtype, represents a personalised medicine approach for the treatment of insulin resistance and T2D.

1.7.4. Proof of principle: Inflammation-induced cellular “insulin resistance”

As proof of principle, a GES for TNFα-induced insulin resistance was recently developed [141]. Using 3T3-L1 adipocytes as the cell-based model, 3325 genes were identified whose expression was altered by the induction of insulin resistance (measured as impaired insulin-stimulated 2-deoxyglucose uptake) by TNFα. Of those genes, only 1022 showed altered expression by the reversal of insulin resistance with a combination treatment of aspirin and troglitazone. From those
1022 genes, a set of 5 genes were selected whose combined expression profile gave the highest predictive power to differentiate the insulin resistant state from the re-sensitised state.

As described above, GESs may be used for screening of patients with T2D. The in vitro-derived GES for TNFα was assessed for its ability to characterise insulin resistant subtypes in a human cohort. This analysis utilised lymphocytes expression data from the San Antonio Family Heart Study [150], and measured the expression of 47,289 transcripts in 1,240 individuals from 42 extended families [151]. The TNFα GES of 5 genes was detected in the human profile dataset, and GES score assigned – comprising the sum of the absolute values of the standardised expression units of each of the 5 genes. This was tested for association with every phenotypic measure available for each subject. Those patients whose expression profiles were most similar to the TNFα GES showed a higher degree of insulin resistance as indicated by the HOMA-IR score (P<0.001) [141]. This correlation is consistent with the use of GES technology to characterise an insulin resistant subtype in this population.

In vitro screening of compound libraries has also been used in this model, assessing the ability of a given compound to affect the genes identified in the GES. Screening for those compounds whose effects on the target genes mirrored the expression profile observed in the “recovered from disease” GES was successful in identifying known insulin sensitising agents such as non-steroidal anti-inflammatory agents, β-adrenergic antagonists, beta-lactams and sodium channel blockers [141]. In addition, a number of chemical families without previously known insulin-sensitising actions were identified including β-lactams, sulfamide antifolates and
carbonic anhydrase inhibitors [141]. The carbonic anhydrase inhibitor methazolamide was identified in the original GES screen, and has since been characterised as a novel insulin sensitiser [149]. In DIO mice, methazolamide treatment lowered both plasma glucose and insulin during a GTT. In obese diabetic \( db/db \) mice, methazolamide lowered fasting blood glucose and HbA\(_{1c}\), and suppressed hepatic glucose production, and furthermore, enhanced the \textit{in vivo} effects of metformin [149].

Investigation of the GES genes and their role in insulin resistance has also yielded positive outcomes. We conducted a series of studies to assess what role (if any) the GES genes might play in the development of insulin resistance. Our investigation of the GES gene STEAP4 was mirrored by the results of data published at that time which showed that STEAP4 protects against inflammation and metabolic dysfunction [140]. This highlighted the utility of the GES in gene discovery related to the particular biological state being investigated, and was further proof of the power of this technique in investigating disease states.

\subsection*{1.7.5. Identification of PA-derived GES from liver cells}

The PA-derived GES once identified can be used for the stratification of patient cohorts as described above. We propose that a PA-derived GES could identify an insulin resistant subpopulation from such a cohort. A key comparison with the different GES models will be the identity of the subgroups identified, and the degree of overlap (if any) observed in the groups. Drug screening, as well as
investigation of the GES genes will also be performed following development of a PA-derived GES.

1.8. Summary

The use of ‘omics’ style approaches to multifactorial disease states such as T2D are becoming increasingly accepted as one way research should investigate these diseases in the 21st century. The success of GES technology in the cancer field as both a diagnostic tool and a drug discovery tool is becoming increasingly apparent, and we have shown this technology is equally applicable to the study of T2D. As disease research is progressing towards the development of personalised medicine as the ‘holy grail’ for treatment regimes, we foresee a future where personalised medicine is seen as the gold standard for patient care. We believe GES technology will provide a platform for the development of novel, personalised treatments for patients with T2D. Further, the data collected via microarray analysis has additional utility via GSEA. By combining this approach with the model development described above, we can investigate regulated pathways at the transcriptional level in both the induction and reversal of lipid-induced dysregulated glucose production – such analysis has rarely been performed before, and has the potential for enhancing our understanding of the processes involved in the restoration of glucose homeostasis – which may be distinct from the processes involved in the induction of such a state. Such findings have significant implications on the methods used to identify novel targets for drug development.
1.9. Aims

The overall aim of this project was to interrogate lipid-induced insulin resistance in a hepatic *in vitro* cell culture system via a global unbiased approach. The specific aims of this study were as follows:

(i) To generate a model of PA-induced, and drug restored, insulin resistance in the liver using the rat-derived FAO hepatoma cell line,

(ii) To generate a microarray-based expression profile of the model,

(iii) To utilise DLDA to identify candidate genes for creation of a PA-based GES,

(iv) To confirm the expression levels of the candidate genes using RT-PCR and in turn, in human lymphocyte tissue to determine whether the PA-based GES characterises individuals with markers of the metabolic syndrome,

(v) To interrogate the gene expression profiles for regulated pathways,

(vi) To generate a comprehensive mass-spectroscopy based lipid profile in order to characterise the effects of PA-induced, and drug restored, insulin resistance on lipid metabolism,

(vii) To confirm the metabolic regulation observed in pathway analysis by measuring the effects of PA-induced, and drug restored, insulin resistance on cholesterol and bile acid levels.
Chapter 2 – *In vitro* model development of hyperlipidemia-induced dysregulation of glucose homeostasis

2.1. Introduction

2.1.1. Modelling dysregulated glucose homeostasis

Induction of free fatty acid-induced insulin resistance *in vitro* has been commonly achieved using the saturated fatty acid PA [41, 152, 153]. Cellular insulin resistance can be defined as a reduction in the normal effects of insulin on any one or more of a range of cellular metabolic processes. The induction of PA-induced insulin resistance in muscle cells has highlighted the role of ceramides [152], DAGs [41] and PKC isoforms (such as PKCε) [154]. The induction of PA-induced insulin resistance in the liver has not been investigated to the same degree, however PKC activation (specifically PKCε) has been implicated in hepatic insulin resistance in *in vivo* modelling using PKCε−/− mice [94].

Despite the success such approaches have had, a limitation in some early modelling attempts was the inference that measurement of decreased Ser473 phosphorylation of Akt was sufficient to identify insulin resistance. It has since become clear that reduced phosphorylation of a single site on a single signalling intermediate such as Akt is insufficient to account for all of the observed intermediary and endpoint defects in insulin action in individuals with T2D [155].

The approach taken in this study was therefore to treat liver cells *in vitro* with PA to
induce insulin resistance, as determined by a key endpoint of insulin signalling – hepatic glucose production. This modelling approach allows for the \textit{in vitro} measurement of defects in the same metabolic pathways that occur in individuals with T2D, and the reversal of which is required to successfully treat the human condition.

\subsection*{2.1.2. Reversing dysregulated glucose homeostasis}

While there have been a number of studies investigating the mechanisms by which individual compounds are able to reverse insulin resistance, these changes are specific to the compound used, and as such it is hard to separate the direct effects of the compound on insulin action versus non-specific effects. Using a combination of multiple compounds, followed by a non-biased whole genome microarray approach, allows for the investigation of the global cellular responses to the reversal of lipid-induced dysregulation of glucose homeostasis. This may yield new information as to the cellular responses for both the induction and reversal of impaired glucose production.

We attempt to reverse PA-induced dysregulation of glucose homeostasis using known anti-diabetic agents from a number of distinct classes. The salicylate class – including acetylsalicylic acid (aspirin) and sodium salicylate – act via inhibition of Ikk\(\beta\) \cite{156} to restore insulin signalling in models of inflammation (TNF\(\alpha\)) induced insulin resistance in liver and adipose tissue \cite{42}. Both aspirin and sodium salicylate have been shown \textit{in vitro} to prevent NF-kB induced transcription when used at 2 mM and above \cite{156}, and are equipotent in their inhibition of Ikk\(\beta\) \cite{157}. Aspirin also possesses potent-acetylation mediated cyclooxygenase (COX) inhibitory
properties, being 100 times more potent towards COX enzyme inhibition than sodium salicylate [158] which lacks the required acetyl group. The contribution of COX activity in the induction of insulin resistance however has been refuted. Specific inhibition of both COX1 and COX2 by ibuprofen and naproxen was shown to be ineffective in restoring TNFα impaired IRS-2 tyrosine phosphorylation in FAO hepatoma cells [42], indicating that it is primarily the inhibition of NF-κB signalling which mediates the effects of aspirin and sodium salicylate on restoring insulin sensitivity.

Although widely used for lowering hepatic glucose production [159], the mechanism of action for the biguanides (including metformin, buformin and phenformin) is still debatable, despite metformin and phenformin being identified as anti-hyperglycaemic agents first in 1957 [160, 161], and buformin the following year in 1958 [162]. Metformin is proposed to activate AMPK by the eNOS-stimulated generation of reactive nitrogen species [163], as well as possessing AMPK-independent mechanisms [164]. In rat H4IIE hepatoma cells, 0.1 – 2 mM metformin-treatment for 18 h increased activation of AMPK, observed as increased Thr172 phosphorylation [165]. Whether this AMPK activation occurs via mitochondrial complex 1 inhibition and subsequent ATP depletion is controversial [165], however this mechanism has been extensively studied. Phenformin is the most potent inducer of ATP depletion, with an IC₅₀ of 13 μM, with buformin having an IC₅₀ of 47 μM and metformin the least potent at 2 mM [166]. The same potency rankings have also been shown for intact cell respiration, dissipation of mitochondrial membrane potential and depletion of reduced glutathione and ROS production in primary human hepatocytes [166]. Interestingly, despite having an IC₅₀ for ATP depletion 150-fold that of metformin, 5 mM phenformin increases
AMPK kinase activity 3-fold higher than 2 mM metformin in H441 lung epithelial cells during the first hour of treatment, and after 24 h treatment remains 2 fold higher [167]. Given the 150-fold difference in ATP depletion, yet modest difference in AMPK activation, the current study will provide evidence as to whether the difference in effect of the biguanides on decreasing glucose production in hepatocytes are due to AMPK dependent or independent mechanisms.

The thiazolidinedione (TZD) class of insulin sensitisers (including rosiglitazone, pioglitazone and troglitazone) are PPARγ agonists which act mainly in the adipose tissue (due to high PPARγ expression), but also in the muscle and liver [168]. Although the three members of this class have potent insulin sensitising effects, the association of serious complications with TZD therapy has resulted in these drugs being withdrawn from sale in many markets [169]. In addition to their PPARγ stimulating activity, both rosiglitazone and pioglitazone have been shown (using protein from rat liver cell lysate) to have binding affinities for numerous proteins regulating mitochondrial function, gluconeogenesis/glycolysis and lipid metabolism, including pyruvate dehydrogenase, glucose-6-phosphate isomerase and acyl-CoA dehydrogenase [170]. Pre-incubation of rosiglitazone (at 10, 30 and 100 μM), troglitazone (at 100 μM) and pioglitazone (at 100 μM) for 10 min inhibited mitochondrial complex 1 activity in rat liver homogenates [171]. Inhibition of mitochondrial complex 1 and subsequent energy dysregulation has been proposed to be one of the mechanism by which TZDs increase insulin-sensitivity. In H-2Kb transgenic mice myotubes (a conditionally immortal cell line derived from transgenic mice harboring a thermolabile simivan virus large tumor antigen regulated by the mouse major histocompatibility complex H-2Kb promoter [172]),
30 min incubation with rosiglitazone has been shown to dose-dependently activate AMPK up to a maximum at 100 – 200 μM [173]. Rosiglitazone has recently been associated with increased risk of cardiovascular disease [25, 174], however it is unclear whether there is a difference between rosiglitazone and pioglitazone on cardiovascular disease [175]. Further, pioglitazone has been recently linked to a small but significant increase to the risk of bladder cancers in adults with T2D [24]. In addition to its PPARγ activation, troglitazone treatment (4 weeks at 400 mg/day) has been shown in obese and diabetic individuals to reduce NF-kB binding activity in nuclear extracts of monocytes, as well as decreasing plasma C-reactive protein (a general marker of inflammation) – indicating troglitazone possesses anti-inflammatory properties [176]. Troglitazone has been associated with cases of drug-induced liver toxicity and hepatitis [177], and as such it is unsuitable for the reversal of insulin resistance in a hepatocyte model as used in this study. Given the increased potency for mitochondrial complex 1 inhibition by rosiglitazone over both pioglitazone and troglitazone, and the potential for fewer adverse cellular effects associated with bladder cancer and liver toxicity, rosiglitazone was chosen for use in this study.

The traditional Chinese medicinal herb *Rhizoma coptidis* has been used for the treatment of diabetes for more than 1000 years [178], however the identification of the active hypoglycaemic agent in these herbs – berberine – was not discovered until the 1980s [179]. The effects of berberine on stimulating glucose uptake into 3T3-L1 adipocytes have been shown to be independent of insulin signalling and involve activation of AMPK [180]. In both insulin sensitive and insulin resistant (24 h treatment with 25 mM glucose and 100 nM insulin) L6 myotubes, post-treatment
with 14 μM berberine for 1 h has been shown to activate AMPK, and improved impaired insulin signalling by increasing GLUT4 translocation to the plasma membrane via increasing insulin-stimulated IRS-1 tyrosine phosphorylation, recruitment of the p85 subunit of PI3K to IRS-1, and increasing Akt Ser473 phosphorylation [181]. Berberine pre-treatment for 30 min at 1 and 10 μM has been shown to reduce IL-6 and TNFα production in HepG2 hepatoma cells following 0.5 mM PA-treatment for 24 h, leading to increased insulin-stimulated glycogen production [182].

The aim of this study was to develop an *in vitro* cellular model of hyperlipidemia-induced insulin resistance, followed by the restoration of the impairment in insulin action by trialling known anti-diabetic and/or insulin sensitising agents. The reversal of the hyperlipidemia-induced impaired glucose metabolism was optimised to use two different classes of anti-diabetic and/or insulin sensitising agents. The use of two different classes is a deliberate feature of the model, so that the regulatory changes in gene expression and metabolism (as measured in Chapters 3-5) represent the reversal of lipid-induced dysregulated glucose metabolism, rather than the effects of one specific compound. The induction of cellular insulin resistance using the fatty acid PA was investigated in both muscle and liver cell lines. While the model was unsuccessfully developed in muscle cell lines (see Appendix 1), PA was successfully used for model development in rat FAO hepatoma cells, as outlined in this chapter.
2.2. Methods

2.2.1. Cell culture
FAO rat hepatoma cells (FAO cells, [183]) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO Invitrogen, Melbourne, VIC, Australia) supplemented with 10% (v/v) Foetal Bovine Serum (FBS, GIBCO Invitrogen) at 37°C and 5% CO₂. Media was replenished every 24 h. FAO hepatoma cells were seeded in 48 well plates at 100,000 cells per well and treatments commenced 24 h post-seeding as previously described [149].

2.2.2. Cell treatments
FAO hepatoma cells were treated with vehicle (RPMI 1640 medium, pH 7.4, 0.0285% (v/v) EtOH, 25μM BSA), 0.075, 0.15 or 0.3 mM PA or linoleate (LA) (RPMI 1640 medium, pH 7.4, 0.0285% (v/v) EtOH, 25μM BSA) for either 24 or 48 h. To induce reversal of the dysregulation of glucose production, a series of ‘reversal agents’ were added in the final 24 h of the 48 h PA treatment. The ‘reversal agents’ were aspirin (1 – 10 mM), sodium salicylate (1 – 10 mM), metformin (0.1 – 1 mM), phenformin (10 – 100 μM), buformin (30 – 300 μM), rosiglitazone (1 – 100 μM) and berberine (1 – 100 μM). Treatment protocol is outlined in Figure 2.1. All treatments in the first 24 h of the 48 h incubation were in normal growth media as described above. In the final 24 h of the 48 h incubation, all treatments were in Glucose Production (GP) media which contained glucose- and serum-free RPMI 1640 media supplemented with 2 mM sodium pyruvate (GIBCO Invitrogen), 20 mM sodium L-
lactate (Sigma-Aldrich, NSW, Australia) and 0.1% (w/v) BSA (USB Corporation) with and without 0.1 nM insulin (Humulin R, Eli Lilly, NSW, Australia).

2.2.3. Measurement of glucose production in FAO hepatoma cells

Glucose content within the conditioned media was measured using a Glucose (GO) assay kit (Sigma), as per manufacturer’s recommendations. Briefly, each sample was diluted 1:2 in dH₂O, then 40 μL of each diluted sample was added to 80 μL of assay reagent in a 96-well plate and incubated at 37°C for 30 min. To stop the reaction, 50 μL of 12 N H₂SO₄ was added to each well, which stabilised the coloration of the o-dianisidine product. Absorbance was read at 540nm on a BioRad xMark Microplate Spectrophotometer using the Microplate Manager 6 software package (BioRad).

Glucose production was corrected by the amount of cellular protein per well measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Briefly, following removal of conditioned media, cells were washed once in 100 μL of 1 x Dulbecco’s Phosphate Buffered Saline, pH 7.4 (1 x PBS), then solubilised in 100 μL of 0.03% sodium-dodecyl sulfate (SDS) for 15 min at room temperature. Each well was mixed via pipetting, then 25 μL of each sample or BSA standard was added to 200 μL of assay reagent (supplied with kit) and incubated for 30 min at 37°C. Absorbance was read at 562 nm as described above, and the absorbance of each sample was compared with the standards to determine protein concentration of each sample.

Due to the high cost of the Glucose (GO) assay kit, the Trinder glucose oxidase method (as described previously [184]) was optimised for use with glucose standards made up in vehicle treatment media (0 – 60 μg/mL glucose) and a series
of volumes of standards were assayed. The maximum volume tested (40 μL from total 200 μL per well) was carried forward for use in the following assays as it showed the steepest trend line, and therefore had the greatest resolution between samples of differing concentrations (data not shown). Briefly, 40 μL conditioned media from each well was added to a 96-well plate and incubated at 37°C for 25 min with 250 μL Assay Buffer (dH2O, pH 7.0, 0.12 M NaH2PO4·2H2O (BDH Chemicals, Kilsyth, VIC, Australia), 0.1% (w/v) detached phenol crystals (Sigma-Aldrich), 0.5 mg/ml 4-aminoantipyrine (MPBio), 1.6 U/ml peroxidise (Sigma) and 10 U/ml glucose oxidase (Sigma)). Absorbance was read at 490nm on a BioRad xMark Microplate Spectrophotometer using the Microplate Manager 6 software package (BioRad). Glucose production was corrected by the amount of cellular protein per well measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

2.2.4. Cellular viability and cytotoxicity measurements in response to lipid and drug treatments

Mitochondrial dehydrogenase function was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) colourmetric method as previously described [185]. This method measures mitochondrial oxidative capacity as a proxy measure of cellular viability [185]. Briefly, 100 μL MTT assay buffer containing 0.5 mg/ml thiazolyl blue tetrazolium bromide (MTT, M2128 Sigma) in serum-free RPMI medium was added to each well of a 48-well plate containing treated cells as described above. Following incubation at 37°C for optimal colour development (for FAO hepatoma cells, 30 min), 500 μL
0.04 M HCl in isopropanol was added to each well to stop the reaction. The blue MTT formazan product was dissolved by mixing the contents of each well via pipetting repeatedly and 200 μL transferred to a 96-well plate for the absorbance to be read at 570nm with 630nm reference wavelength.

Cellular cytotoxicity was measured using a CytoTox 96 ® Non-Radioactive kit (G1780 Promega), as per manufacturer’s recommendation. Briefly, following cellular treatments, conditioned media was collected from each well (for released extracellular LDH measurement) and stored at 4°C until ready to use. To each well, 200 μL of 1 x PBS pH 7.4 was added and cells were freeze thawed twice at -80°C for 30 min to lyse cells and release intracellular LDH. Cell lysate was diluted 1:5 in 1 x PBS pH 7.4, and conditioned media was diluted 1:10 in 1 x PBS pH 7.4. 50 μL of diluted cell lysate or conditioned media was added to 50 μL of assay reagent (supplied with kit), covered to protect from light, and incubated for 30 min at room temperature. 50 μL of stop reagent (supplied with kit) was added to each reaction, then absorbance was measured at 490 nm. Cytotoxicity was determined as the ratio of conditioned media absorbance (extracellular LDH) to cell lysate absorbance (intracellular LDH), and expressed as percentage of vehicle-treated cells.

2.2.5. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM). Data were first analysed for normality using a one-sample Kolmogorov-Smirnov test. All data was determined to be normally distributed. Data were then analysed using independent samples Students T-test, or a one-way ANOVA. Homogeneity of variance was determined using Levene’s Test, and post-
hoc analysis of ANOVA used either Fisher’s least significant difference (LSD, for homogeneous variance) or Games-Howell (for non-homogeneous variance). Data were considered significant at $p<0.05$. All data presented as mean ± SEM unless otherwise stated.

**FIGURE 2.1:** Schematic representation of the 48 h treatment regime for the induction and reversal of PA induced dysregulation of glucose production. Treatment regime for the induction and reversal of the dysregulation of glucose production in FAO hepatoma cells. PA treatment for 48 h induced dysregulated/elevated glucose production. Increased glucose production was reversed with the addition of a series of “reversal agents” in the final 24 h of PA treatment.
2.3. Results

2.3.1. Effects of PA- and LA-treatment on glucose production in FAO hepatoma cells

The effects of PA and LA on glucose production in FAO hepatoma cells were first assessed after 24 h incubation. Primary rat hepatocytes isolated from animals fed a high fat diet rich in canola oil (high in linoleate) for 3 weeks have previously been shown to exhibit increased glucose production and reduced insulin sensitivity compared with hepatocytes taken from animals on a control diet [186], making linoleate of potential use if PA-induced impairment of insulin action could not be successfully modelled (as observed in muscle cell experiments, see Appendix 1). Rat derived FAO hepatoma cells were selected for use in these experiments due to increased glucose production in basal and insulin stimulated conditions compared to alternative hepatic cell models such as the human HepG2 cells (N. Konstantopoulos unpublished in house data) or the FAO hepatoma genetic precursor cell line H4IIE cells [183]. Because FAO hepatoma cells exhibit relatively high glucose production, which can be modulated by fatty acids and drug treatments, they are commonly used for in vitro modelling of hepatic insulin resistant states [42].

Glucose production was measured after 24 h treatment with 0.075, 0.15 and 0.3 mM PA at 37°C in the presence or absence of 0.1 nM insulin. In vehicle-treated cells, insulin treatment suppressed glucose production by -34 ± 9% (p=0.00007, Fig. 2.2 a). PA at 0.075 and 0.15 mM increased basal glucose production by 27 ± 2% and
34 ± 4% respectively (p=0.0005 and p=0.00006 versus basal vehicle-treated, Fig. 2.2 a), but did not significantly alter insulin-suppressed glucose production. In contrast, 0.3 mM PA did not affect basal glucose production nor insulin-suppressed glucose production.

Glucose production was measured after 24 h treatment with 0.075, 0.15 and 0.3 mM LA. In vehicle-treated cells, 0.1 nM insulin for 24 h suppressed glucose production by -28 ± 9% (p=0.0022, Fig. 2.2 b). LA-treatment at 0.075 mM did not alter either basal or insulin suppressed glucose production. LA-treatment at 0.15 mM increased basal glucose production by 27 ± 8% (p=0.0027 versus basal vehicle-treated, Fig. 2.2 b), however insulin-suppressed glucose production was not altered. In contrast, 0.3 mM LA did not alter basal or insulin-suppressed glucose production, similar to the observation in PA-treated cells. As observed for the 24 h PA-incubation, the data show that LA treatment for 24 h did not affect insulin sensitivity, despite increasing basal glucose production at 0.15 mM, therefore the treatment duration was extended to 48 h (see below).
FIGURE 2.2: Dysregulation of glucose production by 24 h treatment with PA or linoleate in FAO hepatoma cells.

(a) Basal (black bars) and insulin suppressed (0.1 nM insulin, white bars) glucose production following 24 h treatment with PA. The average amount of vehicle-treated, basal glucose production was 37.54 ± 1.01 μg glucose / mg protein. (b) Basal (black bars) and insulin suppressed (0.1 nM insulin, white bars) glucose production following 24 h treatment with linoleate. Vehicle-treated, basal glucose production was 52.36 ± 2.58 μg glucose / mg protein. Data are expressed as mean ± SEM. *p<0.05 when compared with basal, vehicle-treated cells. †p<0.05 when compared with each individual treatment’s basal-treated cells. Each experiment was performed once in triplicate.
After 48 h vehicle treatment, 0.1 nM insulin (added in the final 24 h of treatment) suppressed glucose production by -46 ± 6% (\(p=0.0002\) versus basal vehicle-treated, Fig. 2.3 a). PA-treatment at 0.075 and 0.15 mM for 48 h did not affect basal glucose production, while the effects of 0.1 nM insulin were ablated (Fig. 2.3 a). This was observed with a slight but significant decrease in cellular viability of -6 ± 1% (\(p=0.0066\)) and -5 ± 1% (\(p=0.0074\)) respectively compared with vehicle-treated cells, as measured by cleavage of MTT in the mitochondria (Fig. 2.3 c).

Due to variability in glucose production following LA-treatment, the 0.1 nM insulin-suppressed glucose production of -36 ± 7% observed in the vehicle-treated cells was not significant (\(p=0.14\), Fig. 2.3 b). Glucose production was not altered by either 0.15 or 0.3 mM LA-treatment for 48 h compared to vehicle-treated cells. This was observed with an increase in MTT cleavage in the mitochondria at both 0.15 mM (+56 ± 2%, \(p=3.47x10^{-8}\)) and 0.3 mM (+55 ± 2%, \(p=3.66x10^{-8}\), Fig. 2.3 d), indicating an up-regulation of mitochondrial metabolism. LA-treatment therefore did not alter insulin sensitivity in these cells, but did have a significant impact upon mitochondrial oxidative capacity. Hence, PA rather than LA in FAO cells effectively impaired insulin’s ability to reduce glucose production with minimal impairment of cellular viability.
Chapter 2: 

In vitro model development

(a) Glucose Production (% of Vehicle)

(b) Glucose Production (% of Vehicle)

(c) MTT Cleavage (% of Vehicle)

(d) MTT Cleavage (% of Vehicle)
FIGURE 2.3: Dysregulation of glucose production and cellular viability by 48 h treatment with PA or linoleate in FAO hepatoma cells. (a) Basal (black bars), 0.01 nM (dark grey bars), 0.05 nM (light grey bars) and 0.1 nM insulin stimulated (white bars) glucose production following 48 h treatment with PA. Vehicle-treated, basal glucose production was 20.32 ± 1.24 μg glucose / mg protein. (b) Basal (black bars), 0.01 nM (dark grey bars), 0.05 nM (light grey bars) and 0.1 nM insulin stimulated (white bars) glucose production following 48 h treatment with LA. Vehicle-treated, basal glucose production was 20.76 ± 1.07 μg glucose / mg protein. (c) Effect of PA on cellular viability (mitochondrial oxidative capacity) after 48 h treatment. (d) Effect of LA on cellular viability (mitochondrial oxidative capacity) after 48 h treatment. Data are expressed as mean ± SEM. *p<0.05 when compared with basal, vehicle treated cells. †p<0.05 when compared with the relevant basal treated cells. Each experiment was performed three times in triplicate.
FIGURE 2.4: Increased cytotoxicity by 48 h treatment with 0.3 mM PA in FAO hepatoma cells.
Effects of 0.075 - 3 mM PA on LDH ratio after 48 h treatment, expressed as a percentage of the ratio in vehicle-treated cells. The average LDH ratio (extracellular:intracellular) for basal, vehicle treated cells was 0.088 ± 0.003. Data are expressed as mean ± SEM. *p<0.05 when compared with basal, vehicle-treated cells. The experiment was performed once and each treatment measured in triplicate.

LDH release was measured as a marker for cytotoxicity in PA-treated FAO cells. Release of LDH was not significantly increased by either 0.075 or 0.15 mM PA compared with vehicle-treated cells at 48 h (Fig. 2.4). In contrast, 0.3 mM PA for 48 h increased the ratio of LDH release by 148 ± 21% (p=0.00003, Fig. 2.4).
PA-treatment for 48 h at 0.075 mM was selected as the optimal treatment, due to its ability to dysregulate glucose production, while having minimal effects on cellular viability. While 0.15 mM PA treatment was similar in its ability to dysregulate glucose production, the lower concentration of 0.075 mM was chosen in order to mimic the pathological levels of circulating lipids in individuals. Basu et al. have shown that arterial plasma PA concentrations in type 2 diabetic patients range from 175 ± 15 μM after overnight fasting to 75 ± 17 μM following 0.25 mU/kg/min insulin infusion for 3 h, and 38 ± 11 μM following 0.5 mU/kg/min insulin infusion for 3 h [187]. The selected PA concentration of 75 μM falls within this range.

2.3.2. Reversing PA dysregulation of glucose production in FAO hepatoma cells

Compounds with known insulin sensitising and/or anti-diabetes properties were trialled to reverse/restore the PA-induced dysregulation of glucose production in this model. All compounds were assessed for their effects on glucose production when added in the final 24 h of 48 h PA-treatment, and their impact on cellular viability and cytotoxicity was also assessed. The panel of compounds tested included salicylates (aspirin and sodium salicylate), biguanides (metformin, phenformin and buformin), thiazolidinediones (rosiglitazone) and berberine (see Introduction). The data for each experiment is shown in Fig. 2.5, and for ease of comparison has also been summarised in Table 2.1.
TABLE 2.1: Effects of anti-diabetic agents on glucose production, cellular viability (MTT) and cellular cytotoxicity (LDH release) in PA treated FAO cells.

<table>
<thead>
<tr>
<th>Anti-diabetic Agent</th>
<th>Glucose production (% change from PA-treated cells)</th>
<th>Viability (MTT)</th>
<th>Cytotoxicity (LDH)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>Insulin-stimulated</td>
<td></td>
</tr>
<tr>
<td>Salicylates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>1 mM -3 ± 5%</td>
<td>-18 ± 4%</td>
<td>4 ± 2%</td>
</tr>
<tr>
<td></td>
<td>5 mM -46 ± 4%†</td>
<td>-54 ± 4%†</td>
<td>6 ± 1%</td>
</tr>
<tr>
<td></td>
<td>10 mM -86 ± 1%†</td>
<td>-86 ± 1%†</td>
<td>-7 ± 1%†</td>
</tr>
<tr>
<td>Sodium</td>
<td>1 mM -2 ± 5%</td>
<td>-5 ± 5%</td>
<td>2 ± 1%</td>
</tr>
<tr>
<td>Salicylate</td>
<td>5 mM -60 ± 2%†</td>
<td>-66 ± 1%†</td>
<td>5 ± 1%</td>
</tr>
<tr>
<td></td>
<td>10 mM -83 ± 2%†</td>
<td>-85 ± 1%†</td>
<td>-5 ± 3%</td>
</tr>
<tr>
<td>Biguanides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metformin</td>
<td>0.1 mM -5 ± 4%</td>
<td>-9 ± 4%</td>
<td>-2 ± 2%</td>
</tr>
<tr>
<td></td>
<td>0.5 mM -38 ± 2%†</td>
<td>-39 ± 1%†</td>
<td>-1 ± 2%</td>
</tr>
<tr>
<td></td>
<td>1 mM -67 ± 1%†</td>
<td>-69 ± 3%†</td>
<td>-2 ± 3%</td>
</tr>
<tr>
<td>Phenformin</td>
<td>10 μM -33 ± 2%†</td>
<td>-51 ± 1%†</td>
<td>-4 ± 1%</td>
</tr>
<tr>
<td></td>
<td>30 μM -82 ± 1%†</td>
<td>-89 ± 3%†</td>
<td>-2 ± 1%</td>
</tr>
<tr>
<td></td>
<td>100 μM -99 ± 1%†</td>
<td>-97 ± 1%†</td>
<td>-14 ± 1%†</td>
</tr>
<tr>
<td>Buformin</td>
<td>30 μM -37 ± 1%†</td>
<td>-49 ± 2%†</td>
<td>-3 ± 1%</td>
</tr>
<tr>
<td></td>
<td>100 μM -97 ± 1%†</td>
<td>-98 ± 1%†</td>
<td>7 ± 1%†</td>
</tr>
<tr>
<td></td>
<td>300 μM -98 ± 1%†</td>
<td>-97 ± 1%†</td>
<td>-19 ± 1%†</td>
</tr>
<tr>
<td>Rosiglitazone</td>
<td>1 μM -3 ± 4%</td>
<td>-3 ± 5%</td>
<td>-3 ± 2%</td>
</tr>
<tr>
<td></td>
<td>10 μM -9 ± 4%</td>
<td>-14 ± 7%</td>
<td>-3 ± 2%</td>
</tr>
<tr>
<td></td>
<td>100 μM -69 ± 1%†</td>
<td>-81 ± 2%†</td>
<td>17 ± 3%†</td>
</tr>
<tr>
<td>TZD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berberine</td>
<td>1 μM -3 ± 3%</td>
<td>-11 ± 5%</td>
<td>3 ± 2%</td>
</tr>
<tr>
<td></td>
<td>10 μM -22 ± 3%†</td>
<td>-30 ± 5%</td>
<td>18 ± 3%†</td>
</tr>
<tr>
<td></td>
<td>100 μM -81 ± 2%†</td>
<td>-78 ± 1%†</td>
<td>30 ± 5%†</td>
</tr>
</tbody>
</table>

Viability, MTT, % change from PA-treated cells; Cytotoxicity, LDH, % change from PA-treated cells. Data are expressed as mean ± SEM. †p>0.05 versus relevant PA-treated cells. Each experiment was performed three times, in triplicate.
Chapter 2: In vitro model development
FIGURE 2.5: Reversal of PA-induced glucose dysregulation by anti-diabetic agents in FAO hepatoma cells. Basal (black) and 0.1 nM insulin stimulated (white) glucose production following 48 h PA-treatment. (a) Aspirin, (b) sodium salicylate, (c) metformin, (d) phenformin, (e) buformin, (f) rosiglitazone, and (g) berberine were added in the final 24 h of treatment. Basal vehicle-treated glucose production was (a) 33.88 ± 6.13, (b-c) 35.76 ± 1.42, (d-e) 39.62 ± 1.84, and (f-g) 40.95 ± 3.95 μg glucose / mg protein. Data are expressed as mean ± SEM. *p<0.05 for basal PA-treated cells when compared with basal vehicle-treated cells. †p<0.05 for basal PA- and anti-diabetic-treated cells when compared with relevant basal PA-treated cells. ††p<0.05 for insulin stimulated cells when compared with relevant basal treatment. Each experiment was performed three times, in triplicate.
The effects on insulin sensitivity of each anti-diabetic compound were assessed. None of the compounds showed a consistent ability to enhance insulin suppression of glucose production in FAO hepatoma cells treated with 0.075 mM PA for 48 h (Fig. 2.5). Rather, the main effect of each of the seven compounds tested was on basal glucose production. Therefore selection of compounds for the reversal of PA-induced elevated glucose production was determined on their ability to lower basal glucose production to the same level as insulin-suppressed glucose production in vehicle-treated cells.

2.3.2.1. Salicylates

Aspirin was used at 1, 5 and 10 mM in the final 24 h of the 48 h PA-treatment (Table 2.1, Fig. 2.5 a). Aspirin at 5 and 10 mM decreased basal glucose production by -46 ± 4% \( (p=0.00001) \) and -86 ± 1% \( (p=0.0000002) \) respectively compared with basal, PA-treated cells. Both of these concentrations led to increased cytotoxicity, increasing the LDH ratio by 54 ± 11 % \( (p=0.0229) \) and 297 ± 52% \( (p=0.0150) \) respectively when compared with PA-treated cells (Table 2.1). The higher concentration of 10 mM also decreased cellular viability by -7 ± 1%, \( (p=0.0119 \) compared with PA-treated, Table 2.1). The effects of aspirin were therefore observed in parallel with decreased cellular viability at 10 mM, and increased cytotoxicity at 5 and 10 mM.

Sodium salicylate was used at 1, 5 and 10 mM in the final 24 h of 48 h PA-treatment (Table 2.2, Fig. 2.5 b). Compared with aspirin, sodium salicylate showed increased potency at 5 mM, decreasing basal glucose production by -60 ± 2% \( (p=0.000001 \) compared with basal PA-treated cells). Sodium salicylate at this concentration did
not affect cellular viability (Table 2.1). At 5 mM, sodium salicylate increased the LDH ratio by \(73 \pm 13\%\) \((p=0.0236)\) compared with PA-treated cells (Table 2.1). The effects of sodium salicylate at 5 mM were more potent than aspirin, and could be reduced to minimise effects on cytotoxicity. Sodium salicylate was therefore chosen over aspirin as a potential reversal compound.

### 2.3.2.2. Biguanides

Metformin was used at 0.1, 0.5 and 1 mM in the final 24 h of 48 h PA-treatment (Table 2.1, Fig. 2.5 c). Metformin at both 0.5 and 1 mM reduced basal glucose production without altering cellular viability. In addition, metformin at 0.5 and 1 mM improved the LDH ratio, thereby improving the measure of cytotoxicity (Table 2.1). Metformin at 0.5 and 1 mM therefore reduced glucose production in the absence of changes to cellular viability and decreased (improved) cytotoxicity. Due to its current widespread utility in patients, these results confirmed metformin as a candidate reversal compound for PA-induced dysregulation of glucose production.

Phenformin was used at 10, 30 and 100 \(\mu\)M in the final 24 h of 48 h PA-treatment (Table 2.1, Fig. 2.5 d). As previously reported, phenformin showed increased potency over metformin despite being used at a ten-fold lower dose, and also did not affect cellular viability at 10 or 30 \(\mu\)M. However, the improvement to cytotoxicity was not significant with phenformin, and at 100 \(\mu\)M showed a trend for increased cytotoxicity. Buformin was used at 30, 100 and 300 \(\mu\)M in the final 24 h of 48 h PA-treatment and showed similar increased potency for reducing glucose production compared with metformin (Table 2.1, Fig. 2.5 e). Similarly to
phenformin, buformin did not significantly improve cytotoxicity, and also showed a trend for increased cytotoxicity at the higher dose of 300 μM (Table 2.1). Metformin was chosen as a reversal agent for further testing over phenformin and buformin despite the increased potency of phenformin and buformin on lowering glucose production. This was due to the discontinued use of buformin and phenformin in human patients due to concerns over possible association with the induction of lactic acidosis [188, 189], with the aim of making the combination treatment as physiologically relevant as possible.

### 2.3.2.3. Thiazolidinediones

Rosiglitazone was used at 1, 10 and 100 μM in the final 24 h of 48 h PA-treatment (Table 2.1, Fig. 2.5 f). At 1 and 10 μM, rosiglitazone had no effect on glucose production, cellular viability or cytotoxicity. Rosiglitazone at 100 μM decreased basal glucose production by -69 ± 1% (p=2.5x10^{-10} compared with basal PA-treated cells). The decrease in basal glucose production at 100 μM was observed with an increase in cellular viability of 17 ± 3% (p=0.00002 compared with PA treated cells, Table 2.1). Only at the highest dose tested was rosiglitazone able to lower glucose production in PA-treated FAO hepatoma cells, and this occurred with positive effects on cellular viability, however it remains to be observed if this effect would remain at lower concentrations.

### 2.3.2.4. Berberine

Berberine was used at 1, 10 and 100 μM in the final 24 h of 48 h PA-treatment (Table 2.1, Fig. 2.5 g). Berberine at 10 and 100 μM decreased basal glucose
production by -22 ± 3% and -81 ± 2% \( (p=0.0002 \text{ and } p=4.4 \times 10^{-12}) \text{ respectively compared with basal PA-treated). At both 10 and 100 \( \mu \text{M}, \) berberine improved cellular viability by 18 ± 3% and 130 ± 5% respectively \( (p=0.0060 \text{ and } p=0.0020) \text{ compared with PA-treated cells, Table 2.1). Berberine had no effect on cytotoxicity at 1 or 10 \( \mu \text{M}, \) however 100 \( \mu \text{M} \) increased the LDH ratio by 42 ± 10 % \( (p=0.0021) \text{ compared with PA-treated} \) indicating increased LDH release due to a decrease in plasma membrane integrity (Table 2.1).

Based upon the results gained in the single compound experiments, metformin and sodium salicylate were chosen for use in combined therapy. Briefly to summarise their effects, both metformin and sodium salicylate reduced basal glucose production, coupled with minimal effects on cellular viability and cytotoxicity at the lowest (1 mM sodium salicylate and 0.1 mM metformin) and intermediate (5 mM sodium salicylate and 0.5 mM metformin) concentrations tested. Sodium salicylate was selected in preference over aspirin due to increased potency for lowering glucose production at 5 mM. Metformin was used in preference over buformin and phenformin (which were both more potent at lower concentrations) due to metformin’s ongoing utility in human patients [188, 189] – with the aim of making the treatments relevant to the human condition. Given the recent removal of rosiglitazone from sale in multiple countries [190], alongside stringent restrictions on use in the United States of America [191], it was also not utilised for further experimentation. Berberine was able to reduce basal glucose production at the higher concentration tested, however this occurred with large changes to cellular viability and cytotoxicity, and as such berberine was not carried forward for further use.
2.3.4. Optimising combination treatment to reverse PA dysregulation of glucose production in FAO hepatoma cells

For combination treatment, metformin was used at 0.1 - 0.5 mM and sodium salicylate at 1 - 5 mM in the final 24 h of 48 h PA-treatment. As no insulin sensitising effect was observed with either of these compounds on their own, the focus of the reversal shifted away from attempting to improve insulin action, to lowering basal glucose production in PA-treated cells to the same level observed in insulin-stimulated, vehicle-treated cells. In addition to the ability to reduce glucose production, it was also determined whether any cumulative effect could be achieved with combination therapy, or whether there was a dominant glucose lowering effect by a single agent. The results of the reversal experiment which successfully reduced the PA-induced increase in glucose production to the same level as insulin-stimulated, vehicle-treated cells is summarised in Table 2.3 below.

For the complete dataset for each combination reversal experiment, see Appendix 2.
TABLE 2.3: Reversal of the PA-induced increase in basal glucose production using co-treatment with metformin and sodium salicylate.

<table>
<thead>
<tr>
<th>Sodium Salicylate</th>
<th>Metformin</th>
<th>Basal Glucose production (% change from PA-treated cells)</th>
<th>Viability (MTT, % change from PA-treated cells)</th>
<th>Cytotoxicity (LDH, % change from PA-treated cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>0 mM</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.25 mM</td>
<td>0.25 mM</td>
<td>-21 ± 4%†</td>
<td>-13 ± 1%†</td>
<td>-7 ± 4%†</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.25 mM</td>
<td>-22 ± 5%†</td>
<td>-31 ± 3%†</td>
<td>-4 ± 2%</td>
</tr>
<tr>
<td>2 mM</td>
<td>0.25 mM</td>
<td>-42 ± 4%†</td>
<td>-47 ± 3%††</td>
<td>-9 ± 1%†MN</td>
</tr>
<tr>
<td>3 mM</td>
<td>0.25 mM</td>
<td>-54 ± 2%†</td>
<td>-56 ± 3%††</td>
<td>-2 ± 1%†</td>
</tr>
<tr>
<td>4 mM</td>
<td>0.25 mM</td>
<td>-65 ± 2%†</td>
<td>-66 ± 3%††</td>
<td>+8 ± 1%†MN</td>
</tr>
<tr>
<td>5 mM</td>
<td>0.25 mM</td>
<td>-58 ± 2%†</td>
<td>-69 ± 2%††</td>
<td>+11 ± 1%†MN</td>
</tr>
</tbody>
</table>

Effects of metformin and sodium salicylate on glucose production, cellular viability (MTT) and cellular cytotoxicity (LDH release) in PA-treated FAO cells. † p<0.05 versus PA-treated cells. M p>0.05 versus metformin-alone treated cells. N p>0.05 versus sodium salicylate alone treated cells. Data are represented as mean ± SEM of three independent experiments performed in triplicate.

Insulin suppressed glucose production in vehicle-treated cells by -38 ± 4% (p=0.0002 compared with basal vehicle-treated cells), consistent with previous results, while PA-treatment raised basal glucose production by 28 ± 3% (p=0.0014) compared with basal vehicle-treated cells (see Appendix 2, Appendix Fig. a2.2). The optimal combination therapy was required to reduce basal glucose production to the same level as observed in insulin-stimulated, vehicle-treated cells. For comparative purposes, this equates to a reduction of 51% from basal PA-treated cells. The combination which best achieved this was 0.25 mM metformin and 2 mM sodium salicylate, at -47 ± 3% (p=1.8x10^-7 compared with basal, PA-treated cells), and was not significantly different to insulin-suppressed, vehicle-treated cells (p=0.999).
Combination treatment with 0.25 mM metformin and 2 mM sodium salicylate caused a decrease in cellular viability of -7 ± 1% compared with PA-alone treated cells ($p=0.00001$, Table 2.3). The combination treatment had no effect on cytotoxicity as measured by LDH ratio compared with PA-alone treated cells (Table 2.3).

Metformin at 0.25 mM and sodium salicylate at 2 mM therefore successfully reversed the PA-induced dysregulation of glucose production. Although an additive effect was not observed with these concentrations, both metformin at 0.25 mM and sodium salicylate at 2 mM lowered glucose production independently, and the combination reduced glucose production to the same level as observed in insulin-stimulated, vehicle-treated cells.

### 2.3.5. Metformin and sodium salicylate reverse PA dysregulation of glucose production in FAO hepatoma cells

In order to fully characterise the effects of metformin and sodium salicylate on PA-induced dysregulation of glucose production, the selected concentrations of 0.25 mM metformin and 2 mM sodium salicylate were repeated eight independent times to set stringent criteria for the selection of samples to be used in microarray analysis (see Chapter 3). Insulin suppressed glucose production in vehicle-treated cells by -34 ± 1% ($p=6.6\times10^{-13}$, Fig. 2.6 a). PA-treatment for 48 h raised basal glucose production by 25 ± 2% ($p=0.00002$ compared with basal, vehicle-treated cells), while the insulin effect on glucose production in PA-treated cells was completely ablated. Combination treatment using 0.25 mM metformin and 2 mM sodium
salicylate (PAMN) lowered basal glucose production by -45 ± 2% compared with basal, PA-treated cells \((p=7.1\times10^{-13}, \text{Fig. 2.6 a})\). Insulin suppressed glucose production in PAMN-treated cells was significantly lowered by -8 ± 2\% \((p=0.018, \text{Fig. 2.6 a})\). Insulin suppressed glucose production in vehicle-treated cells was not significantly different to either basal \((p=0.5269)\) or insulin suppressed \((p=0.2988)\) glucose production in PAMN-treated cells (Fig. 2.6 a).

PA-treatment for 48 h lowered cellular viability by -15 ± 1\% compared with vehicle-treated cells \((p=1.3\times10^{-17}, \text{Fig. 2.6 b})\). PAMN-treatment did not significantly alter cellular viability compared with PA-treated cells \((p=0.10; \text{Fig. 2.6 b})\). Similarly, PA-treatment increased the LDH ratio by 15 ± 4\% compared with vehicle-treated cells \((p=0.021, \text{Fig. 2.6 c})\), however PAMN-treatment did not further change LDH ratio compared with PA-treated cells \((p=0.25, \text{Fig. 2.6 c})\). As the PAMN-treatment did not further impair cellular viability or cytotoxicity, it was concluded that the reduction in basal glucose production observed with PAMN treatment is a glucose lowering effect, and not due to a side effect of impaired cellular function with the combination treatment.
Chapter 2: In vitro model development

a

Glucose produced (% of Vehicle)

Vehicle 0.075 mM PA 0.075 mM PA +0.25 mM Met +2 mM NaS

b

MTT Cleavage (% of Vehicle)

Vehicle 0.075 mM PA 0.075 mM PA +0.25 mM Met +2 mM NaS

c

LDH Ratio (% of Vehicle)

Vehicle 0.075 mM PA 0.075 mM PA +0.25 mM Met +2 mM NaS
FIGURE 2.6: Combination reversal of PA-induced glucose dysregulation using 0.25 mM metformin and 2 mM sodium salicylate in FAO hepatoma cells.

(a) Basal (black bars) and 0.1 nM insulin stimulated (white bars) glucose production following 48 h treatment with PA, with metformin and sodium salicylate added in the final 24 h of treatment. The level of glucose produced by basal, vehicle-treated cells was equal to 50.03 ± 3.86 μg glucose / mg protein. (b) Effect of PA, metformin and sodium salicylate on cellular viability after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of the 48 h PA-treatment. (c) Effects of PA, metformin and sodium salicylate on LDH ratio after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of the 48 h PA-treatment. Average LDH ratio (extracellular:intracellular) for basal, vehicle-treated cells was 0.110 ± 0.009.

Data are expressed as mean ± SEM. *p<0.05 for basal PA-treated cells when compared with basal, vehicle-treated cells. †p<0.05 for basal PAMN-treated cells when compared with basal PA-treated cells. ††p<0.05 for insulin stimulated cells when compared with each individual treatment’s basal treated cells. (a) Average of 8 independent experiments performed in triplicate; (b-c) average of six experiments in triplicate.
2.4. Discussion

In contrast to the *in vitro* muscle cell models (see Appendix 1), the liver cell model was superior in its ability to demonstrate the induction and reversal of PA-induced dysregulation to a key endpoint of insulin action. Firstly, 0.1 nM insulin suppression of glucose production for 24 h in FAO hepatoma cells was consistently greater than 30%. This contrasts favourably over the muscle cell models whereby the maximum insulin effect was observed to be 27 ± 1% in HA-GLUT4 translocation in stable transfected L6 cells, and was lower in the L6 and C2C12 cell lines used to measure 2-deoxyglucose (2-DOG) uptake and glycogen synthesis respectively. Secondly, the induction of cellular insulin resistance was demonstrated with 75 – 150 μM PA for 48 h, after which insulin suppression of glucose production was ablated. Again, this contrasts to the muscle cell models, where complete ablation of the insulin response was frequently observed in parallel with significant decreases in basal measurements – suggestive of reduced cellular viability (although this was not measured in the muscle cell models). Finally, the induction of dysregulated glucose production in FAO hepatoma cells with PA-treatment occurred in parallel with mild changes to both cellular viability and cytotoxicity, which were not further affected in the reversal with PAMN-treatment. Therefore, the observed regulation of glucose production with PA, metformin and sodium salicylate were due to the effects of the lipid or compound on glucose metabolism, rather than off-target effects due to compromised cellular integrity. In addition, there is currently less understood about the induction of PA-induced insulin resistance in the liver than in muscle tissue, and as such this model has greater potential to provide novel information.
All seven of the compounds tested were able to reduce basal glucose production to varying degrees (Table 2.2). The ability of each compound to reverse the PA-induced increase in basal glucose production yields potential mechanisms of action for the effects of PA in these cells. The ability of both sodium salicylate and aspirin to lower basal glucose production may indicate a potential for an underlying inflammatory mechanism of action with PA-treatment [156]. The induction of insulin resistance by PA in hepatocytes has previously been shown to involve activation of the NF-kB pathway [192], and therefore the inhibition of this signalling pathway by the salicylates is consistent with the effects observed on glucose production in this model. Berberine is also a suppressor of inflammation, and has been shown in 3T3-L1 adipocytes to suppress the gene expression levels of TNFα and IL-6 [193]. In human HepG2 hepatoma cells, berberine was demonstrated to lower PA-induced increases in the excreted protein level of these 2 inflammatory cytokines in conditioned media [182], alongside restoration of PA-inhibited insulin-stimulated glycogen synthesis. Both aspirin and sodium salicylate lowered basal glucose production only at a concentration greater than 1 mM, keeping with the established inhibition of NF-kB signalling and cytokine production at 2 mM and above [156]. Aspirin, but not sodium salicylate, increased insulin sensitivity at the lower concentration of 1 mM (Fig. 2.5 a), and given the increased potency for COX inhibition by aspirin [158], this finding may indicate that COX inhibition is an effective mechanism for the restoration of insulin sensitivity in this model. This effect however was not observed at 5 and 10 mM, indicating the dominant effect of NF-kB inhibition in lowering basal glucose production at these concentrations.
The reduction in glucose production by metformin does not suggest a mechanism for the effects of PA on glucose production. There are multiple proposed mechanisms of action for metformin in the liver. The main proposed mechanisms of action for metformin involves AMPK activation via the generation of reactive nitrogen species [163], and may involve downstream induction of Sirt1 activity [194]. It has also been reported however that metformin can act independently of AMPK to reduce glucose production, although the mechanisms by which this is achieved are not completely understood [164]. PA-treatment does not affect the activation of AMPK [195], and thus the actions of metformin do not restore a PA-induced defect. Interestingly, phenformin and buformin showed a greater than ten-fold potency for lowering glucose production than metformin. Given the relative similarity in AMPK activation that others have reported for metformin and phenformin [167], it is likely that the difference in potency observed between phenformin, buformin and metformin are due to AMPK independent mechanisms.

The main actions of rosiglitazone on glucose production in the liver, similarly to metformin, are likely to be independent of the PA-induced defects – although rosiglitazone has been shown to reduce mononuclear blood cell NF-kB activation and plasma TNFα levels in patients [196]. Activation of PPARγ in adipose tissue is thought to lead to increased expression of adiponectin, an adipokine which activates AMPK. It has been reported that troglitazone (another TZD) induces rapid AMPK activation in muscle, liver and adipose tissue independently of adiponectin expression [197]. The concentrations of rosiglitazone used here correlate with its ability to inhibit mitochondrial complex 1 [171] and activate AMPK [173]. It is
therefore likely that rosiglitazone is acting independently of the mechanisms by which PA-induces dysregulated glucose production in the current study.

Any conclusions as to the mechanism of action in this model based on the compounds used however has to consider the inability of any of the compounds to reverse the PA-induced cellular insulin resistance. It is therefore likely that the effects of PA on inducing insulin resistance and elevated glucose production occur by independent mechanisms to which the lowering of basal glucose production is achieved by treatment with the compounds. This will be further investigated in Chapter 4, where the effects of PA treatment on the alteration of whole genome gene expression will be compared with the effects of metformin and sodium salicylate treatment on whole genome gene expression. Of course, this finding has significant implications for the main aims of the project. Therefore, this study does not model the induction and reversal of PA-induced insulin-resistance. The updated aim for the project is to model the PA-induced dysregulation of glucose production in the liver (which includes the induction of insulin resistance and increased basal glucose production) and the reversal of such a state (which involves the lowering of basal glucose production, but not necessarily the restoration of insulin sensitivity).

Therefore, a key finding described in this chapter is the inability of any of the compounds tested to improve insulin sensitivity in PA-treated FAO hepatoma cells. This is in contrast with the effects of these compounds in other cell types, or against other insults, where they have been shown to improve insulin sensitivity. For example, aspirin has been shown \textit{in vivo} to improve whole body insulin sensitivity in Zucker\textsuperscript{fa/\textit{fa}} rats and leptin\textsuperscript{ob/ob} mice [42], both models of obesity and insulin
resistance. The same study also showed that pre-treatment of both aspirin and sodium salicylate can protect from insulin-responsive IRS-2 tyrosine phosphorylation defects induced by TNFα treatment in FAO cells. Aspirin also protected against TNFα-induced IRS-1 tyrosine phosphorylation defects in 3T3-L1 adipocytes [42]. However it was not described if these changes at the insulin signalling level resulted in a rescue of altered glucose metabolism in these cells.

Metformin has been previously reported to improve hepatic insulin sensitivity in T2D patients [143, 198], as well as lowering basal glucose production [199]. Contrasting this, other studies have reported that metformin acts mainly by lowering basal glucose production alone [200]. Regardless, the lowering of basal glucose production is a common finding amongst all human studies with metformin in diabetic patients, and is consistent with the findings presented here. It is perhaps possible that by lowering basal hepatic glucose production, and subsequently lowering plasma glucose levels, a reduction in the secretion of insulin from pancreatic β-cells is achieved – preventing β-cell exhaustion and improving insulin sensitivity due to lower plasma insulin levels maintaining glycaemic control.

Rosiglitazone has been shown to improve whole body insulin sensitivity in C57BL/6 mice fed a corn oil-based high-fat diet [201]. Although rosiglitazone’s major target organ is the enriched PPARγ-expressing adipose tissue, rosiglitazone has been shown to reduce basal glucose production and increase hepatic insulin sensitivity in human type 2 diabetic patients [202]. This is in contrast with the results obtained in this study, however it is possible that the systemic effects of whole body rosiglitazone treatment may elicit responses not seen in individual cellular
experiments as reported in this study (as with metformin). For example, activation of PPARγ has an inhibitory effect on the expression of pro-inflammatory cytokines such as TNFα [203]. By activating PPARγ with rosiglitazone, a reduction in circulating proinflammatory cytokines would reduce systemic inflammation – thereby reducing inflammation-based insulin resistance if present in the liver.

Experiments performed in human HepG2 hepatoma cells showed that pre-treatment with 1 and 10 μM berberine for 30 min could prevent PA-induced insulin resistance developing over 24 h, measured using insulin-stimulated glycogen synthesis [182]. Glycogen synthesis in FAO hepatocytes was difficult to measure directly due to very low levels of intracellular glycogen (unpublished in-house data), thus making a direct comparison across the different cell lines difficult. At the same concentrations however, berberine did not enhance insulin sensitivity in FAO hepatocytes in the presence of PA in this study. Berberine (0.01 – 100 μM for 24 h) has also been shown to increase glucose transport in 3T3-L1 adipocytes in an insulin-independent manner [180]. This is consistent with the effects of berberine reported in this chapter, where the reduction in glucose production was independent of insulin sensitivity.

The main effect of all compounds tested in this study was on basal glucose production. Whether this is a reflection of the unique insulin signalling intermediates of the liver (IRS-1 is the main receptor substrate in the muscle, while IRS-2 is the main receptor substrate in the liver), or lack of systemic factors operating in in vivo models is unclear. While this finding was surprising, the model still remains a valid in vitro version of the human condition, where lowering hepatic
glucose production (with compounds such as metformin) is an important remedy for elevated blood glucose levels.

In summary, we developed an *in vitro* cellular model of hyperlipidemia-induced insulin resistance using the fatty acid PA. While the specific restoration of insulin action in rat FAO hepatoma cells using metformin and sodium salicylate was unsuccessful, the combination treatment was able to reverse the increase in glucose production observed with PA treatment. The remainder of this thesis therefore investigated the induction and reversal of elevated fatty acid induced glucose production, rather than the specific inhibition and restoration of insulin action. Although this avenue of investigation diverged from the original aim, the lowering of hepatic glucose production in the presence of elevated lipids remains clinically relevant to obesity-induced T2D.
Chapter 3 – Identification of a GES that discriminates between dysregulated hepatic glucose production (PA-treated) and restored, healthy hepatic glucose production (PAMN-treated)

3.1. Introduction
Developed nearly two decades ago for the measurement of the expression of multiple genes in parallel [124], microarray technology can now be used to assess the expression of tens of thousands of genes in a sample simultaneously. This provides a powerful tool to assess genome wide transcriptional events for any given cell or tissue, in any biological state. Microarray technology has a range of applications including identifying disease-causing genes, identifying targets for new therapies and prediction of drug responsiveness [125]. The major applications for microarray technologies include identification of differentially expressed genes – the focus of this chapter, and the identification of co-ordinately regulated signalling or metabolic pathways – the focus of Chapter 4.

Initially applied to the study of cancer malignancy, early genomic scale microarray studies allowed for the identification of large profiles consisting of hundreds of differentially expressed genes [204]. While such expression profiles are robust in their ability to characterise different cellular states, the sheer number of genes involved presents a technical challenge in the application of such profiles to characterise patients in a clinical setting. Therefore, the statistical selection of a small subset of genes (termed Gene Expression Signature, GES) that robustly
discriminates between two biological states enhances the utility of such information: through multiplex RT-PCR analysis, the measurement of the selected genes can be quickly, and more cost effectively, determined in patient samples. This can be achieved by minimising the extensive redundancy that exists in such large datasets by using only a relatively small subset of discriminating genes, which can approach equivalence in predictive power to the whole dataset when properly identified [132].

The challenge for the application of a discriminating GES is the availability of relevant patient tissues for ex vivo measurement of the relevant genes. While the GES being developed in this study was generated in hepatoma cells, it would be unrealistic to expect all diabetic patients to undertake liver biopsies for characterisation of their disease. An attractive alternative is the measurement of gene expression in the relatively non-invasive white blood cell population, undertaken as a proxy for hepatic gene expression. Disease specific alterations to gene expression profiles of lymphocytes have been shown previously [205]. The application of lymphocyte gene expression as a marker for disturbed disease states in other tissues has been shown for numerous diseases including Alzheimer’s disease [206], first-episode psychosis [207], rheumatoid arthritis [208] and coronary artery disease [209]. However, the expression profiles from these studies were identified in the lymphocyte tissue – while in this study we are developing the GES in the relevant biological tissue in vitro. The relationship between lymphocyte and liver gene expression in a pre-diabetic animal model has been previously described [148]. In pre-diabetic Otuka Long-Evans Tokushima Fatty rats, 1080 genes were found to be regulated in either lymphocytes or liver tissue compared with control
animals. Of those 1080 genes, 57 were found to be regulated in both lymphocytes and liver tissue. Therefore, while not all gene expression changes in the face of a disease state are likely to change in both tissues, there is at least a subset of genes that shows a common expression profile across the liver and lymphocyte tissues.

The early application of GES in the cancer field was for the characterisation of malignancy in various cancer cell types [131, 210], allowing for better prediction of disease and treatment outcomes in cancer patients [132, 133]. This has recently been applied to the setting of insulin resistance, where a TNFα-based GES was able to identify a subset of individuals with more severe insulin resistance from a larger cohort [141]. In addition, the use of GES in high-throughput screening platforms for the identification of novel therapies from chemical libraries has also been applied to such disease states as cancer [139, 211] and insulin resistance [141, 149].

Therefore, the initial aim of this chapter was to perform microarray analysis of vehicle-, PA- and PAMN-treated FAO mRNA, for further use in interrogating the gene expression changes to signalling and metabolic pathways in Chapter 4. The secondary aim of this chapter was to interrogate the microarray data in an unbiased manner to develop a GES-based screening platform. This involved the identification of the set of genes that is the most discriminating between the PA-treated (dysregulated glucose production) and PAMN-treated (restored glucose production) states. This GES will then have two potential applications: first, to screen a library of compounds to identify new anti-diabetic agents, and second, to stratify a cohort of individuals with varying degrees of insulin resistance to attempt to identify an insulin resistant cohort of patients [141]. Once identified, putative GES genes need
to be validated in two ways. First, the microarray-determined mRNA levels will be confirmed using an alternative technology such as RT-PCR analysis. Secondly, as the genes were identified in an in vitro cell line, the biological relevance of the genes will be determined by measuring their expression in human lymphocytes. The tertiary aim of this chapter, once the biological relevance of the GES genes has been established by assessing their expression in a human lymphocyte gene expression data set (from the San Antonio Family Heart Study (SAFHS) [150] in collaboration with Professor John Blangero and Dr. Tom Dyer at Texas Biomedical Research Institute, San Antonio), is to mine the cohort data to determine if any of the GES genes are associated with metabolic syndrome phenotypes.
3.2. Methods

**FIGURE 3.1: Flowchart for GES development.**
Flowchart of methodology from collection of RNA samples for microarrays and data normalisation through to Diagonal Linear Discriminate Analysis (DLDA) and gene validation.

3.2.1. Selection of samples for microarray analysis

Selection of samples for microarray analysis was based upon statistical confirmation for the following three criteria: (i) a robust reduction in glucose production in vehicle-treated cells by 0.1 nM insulin, (ii) a reduced insulin effect in 0.075 mM PA-treated cells, and (iii) a lowering of basal glucose production in the presence of 0.075 mM PA, 0.25 mM metformin and 2 mM sodium salicylate (PAMN) to within 20% of the levels observed in insulin-stimulated vehicle-treated cells. The average values were as follows: (i) in vehicle-treated cells, insulin resulted in a -34% ± 3% decrease in glucose production ($p=1.21\times10^{-9}$ compared with basal vehicle-treated
cells), (ii) PA-treatment increased basal glucose production by $+32 \pm 3\%$ ($p=5.08\times10^{-8}$ compared with basal vehicle-treated cells) and completely ablated the effects of insulin on glucose production, and (iii) PAMN-treatment lowered basal glucose production to the same level as observed in insulin suppressed vehicle-treated cells ($p=0.1308$). These findings are summarised below in Fig. 3.2.

**FIGURE 3.2: Glucose production for selected microarray samples.** Basal (black bars) and 0.1 nM insulin stimulated (white bars) glucose production following vehicle-, PA- or PAMN-treatment for 48 h. Vehicle treated, basal glucose production was equal to $33.05 \pm 2.52 \mu g$ glucose / mg protein. Data are expressed as mean ± SEM. *$p<0.05$ for basal PA-treated cells when compared with basal vehicle-treated cells. †$p<0.05$ for basal PAMN-treated cells when compared with basal PA-treated cells. ††$p<0.05$ for insulin stimulated cells when compared with each individual treatments basal treated cells. Average of 5 experiments performed in triplicate.
3.2.2. RNA extractions and microarrays

Total RNA was extracted from FAO cells treated with VEH (n=8), PA (n=20) or PAMN (n=20) using TRIzol reagent (Invitrogen), and purified using RNeasy Mini Kit columns (Qiagen, Mannheim, Germany) as per manufacturer’s instructions. Quality and quantity of the purified RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and RNA 6000 Nano Assay Kit (Agilent, Melbourne, Australia). Fluorescently labelled cDNA was prepared from 800 ng of total RNA using Agilent Quick Amp Labeling Kit (5190-0424) and Agilent One Color RNA Spike-In Kit (5188-5282), as per manufacturer’s instructions. The cyanine 3-CTP labelled cDNA was hybridised for 17 h to Agilent Whole Rat Genome (4x44k) Oligo Microarray Slides (G4131F) using Agilent Gene Expression Hybridisation Kit (5188-5242) as per manufacturer’s instructions. Fluorescent images of microarrays were acquired using GenePix 4000B scanner, with data extraction performed via GenePix 5.1 software (Molecular Devices, Melbourne).

Normalisation and primary analysis of microarray data was performed using Acuity 4 software (Molecular Devices) as previously described [212]. Briefly, the fluorescent reading for all duplicate genes were averaged, leaving 37,047 unique array gene identification numbers. Of those, 14,098 genes were detected in each sample (n=20 for PA and PAMN, n=8 for vehicle). Each array dataset sample was normalised so that the median expression value in each array was 1.0. The average %CV for vehicle-treated cells was 39%, for PA-treated cells was 34% and for PAMN-treated cells was 31%. The %CV values for the internal Agilent SpikeIn controls in the linear range for long(signal) against log(relative concentration) were 10% for vehicle, 8% for palmitate and 7% for PAMN. The low relative %CV values for the
internal controls indicates technical proficiency with the microarray experimentation, and the variation observed in sample data is mostly due to intersample biological variation, rather than lack of technical proficiency. Genes were selected for further analysis if they were detected in at least 5 vehicle, 15 PA and 15 PAMN samples, of which 16,276 genes fulfilled the selection criteria.

3.2.3. Diagonal Linear Discriminate Analysis (DLDA) for identification of GES genes

To identify a small subset of genes that best discriminate between PA- and PAMN-treated cells, DLDA was performed using Acuity 4 software [138, 141]. DLDA was performed on the entire normalised data set to select the subset of genes that best discriminated between PA- and PAMN-treated cells. The selected genes best characterised the reversal of the effect of PA-treatment on glucose production, rather than any unrelated effects of the metformin and sodium salicylate treatment alone. Therefore, the DLDA algorithm required genes which were significantly different between vehicle- and PA-treated cells (nominal $p<0.05$), while also being significantly different between PA- and PAMN-treated cells (nominal $p<0.05$). There was no requirement for difference in gene expression levels between vehicle and PAMN-treated cells. Of the 450 genes that showed evidence for significant difference between vehicle- and PA-treated cells, only 75 of these genes were also significantly different between PA- and PAMN-treated cells. The DLDA algorithm then utilised a forward step-wise variable selection process to identify the minimum subset of genes that had the most power to distinguish between both vehicle- and PA-treated, and also between PA- and PAMN-treated cells in the microarray data.
Fourteen genes were identified via the DLDA algorithm, of which 7 were able to be mapped to known genes using BLAST database analysis.

### 3.2.4. RNA extractions and RT-PCR validation of GES genes

Total RNA was extracted and quantified from a randomly selected subset of samples used in microarray analysis treated with vehicle (n=4), PA (n=4) or PAMN (n=4) (technical replicates) and an alternative set of vehicle- (n=6), PA- (n=6) and PAMN- (n=6) treated FAO cells (biological replicates). 1 μg of sample RNA in 10 μL nuclease free water (NFW) was added to 1 μL dNTP mix (10 mM) and 1 μL oligo(dT) primer (0.5 μg/μL), then incubated at 65°C for 5 min, placed on ice for 2 min and briefly centrifuged. To each sample was added 5 μL of 5 x reverse transcription buffer, 2 μL DTT (0.1 M), 1 μL RnaseOUT (40 U/μL), 0.25 μL Superscript III reverse transcription enzyme (200 U/μL) and 0.75 μL NFW. Samples were then heated to 50°C for 50 min, followed by 5 min at 85°C and finally cooled to 4°C. Samples were diluted 1:10 in NFW and stored at -80 prior to use.

Gene expression was analyzed by semiquantitative real-time PCR (RT-PCR) using Brilliant SYBR Master Mix (Stratagene, La Jolla, CA) on the MX3005P QPCR system (Stratagene). Validation was performed on the same RNA used for microarray (technical replication) as well as alternative sets of treated samples (biological replication). The RT-PCR conditions were 95°C for 10 min and 40 cycles of 95°C for 30 s and 60°C for 1 min. Relative gene expression was calculated as $2^{-\Delta Ct}$, where $C_t$ is threshold cycle. RT-PCR results were normalised to cDNA concentration, measured using Quant-iT Oligreen ssDNA Assay Kit (Invitrogen), as per manufacturer’s
instructions. Table 3.1 displays the list of primer sequences. All primers were designed using Beacon Designer 7.2 (Premier Biosoft International, CA, USA).

### TABLE 3.1: Primer sequences for RT-PCR analysis of the seven target genes identified through DLDA.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>NCBI Ref. No.</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxa3</td>
<td>NM_012823</td>
<td>GGAGGAGATGATTGAAGAAGATG</td>
<td>CATTCCGCTTGCCCATTGC</td>
</tr>
<tr>
<td>Slc2a2</td>
<td>NM_012879</td>
<td>CTGGTCTCTGTCTGTGTC</td>
<td>TCCTGATACGCTTCTTCC</td>
</tr>
<tr>
<td>Gstm3</td>
<td>NM_020540</td>
<td>GACACTTTGGAGAACCAG</td>
<td>GACTTCAAGAACTCTGGC</td>
</tr>
<tr>
<td>Pla2g1b</td>
<td>NM_031585</td>
<td>GAGGGAGTACAACAACTACG</td>
<td>CTGATTGTAGCAGTGTC</td>
</tr>
<tr>
<td>Rab33a</td>
<td>NM_001108257</td>
<td>GAGACATCAGCCAAGGAC</td>
<td>GCATCACGGTACAAGAGG</td>
</tr>
<tr>
<td>Serpinsa5</td>
<td>NM_022957</td>
<td>TGCTCCAAGGCTCTATCAC</td>
<td>CAGAATCTCAGTCCAATGTCC</td>
</tr>
<tr>
<td>Snn</td>
<td>NM_001034083</td>
<td>CTGGCTTGGATGTGTAGG</td>
<td>GTCAGTGGATGATGAGAGG</td>
</tr>
</tbody>
</table>

Anxa3, annexin a3/LC3, lipocortin 3; Slc2a2, solute carrier family 2 (facilitated glucose transporter), member 2/GLUT2, Glucose transporter 2; Gstm3, glutathione S-transferase mu 3; Pla2g1b, phospholipase A2, group IB; Rab33a, RAB33A, member RAS oncogene family; Serpinsa5, serpin peptidase inhibitor, clade A, member 5; Snn, stannin.

### 3.2.5. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM). Data were first analysed for normality of distribution using a one-sample Kolmogorov-Smirnov test. Data were then either analysed using independent samples Students T-test, or a one-way ANOVA. Homogeneity of variance was determined using Levene’s Test, and post-hoc analysis of ANOVA used either Fisher’s least significant difference (LSD, for homogeneous variance) or
Games-Howell (for non-homogeneous variance). Preliminary correlations between gene expression data and human phenotype data [141] were determined via Pearson correlation for normally distributed data, or Spearman for non-normally distributed data sets. Where a preliminary correlation was found, Linear Regression analysis was performed to confirm whether an association was independent of age, sex and BMI. Data were considered significant at $p<0.05$. 
3.3. Results

3.3.1. DLDA identified the most discriminating genes between PA- and PAMN-treated cells

From the 450 genes that were identified in the microarray data as differentially expressed between vehicle- and PA-treated cells, 75 of these were also differentially expressed between PA- and PAMN-treated cells. DLDA identified 14 genes from the microarray data that best discriminated between PA- and PAMN-treated cells (Table 3.2). Of the 14 genes, only the genes that could be mapped to the rat genome were chosen for further analysis. This was achieved via the Basic Local Alignment Search Tool (BLAST: http://blast.ncbi.nlm.nih.gov/) as two separate searches: against all *Rattus norvegicus* genes and against all reference RNA sequences optimized for highly similar sequences. There were 6 sequences from the microarray data identified with 60mer microarray sequences which could not be mapped to known *Rattus norvegicus* genes - AA998612, DY472907, TC628671, TC641785, TC612212, TC603506. Of the remaining 8 genes, one was a predicted sequence duplicate of *Pla2g1b*, RDG1560824_predicted phospholipase A2 precursor, which was not carried forward, due to there being no sequence homology to any *Rattus norvegicus* gene for the 60mer sequence from the microarray. The 60mer sequences from the Agilent 4x44k microarray chips for all 14 identified genes are detailed in Appendix 3.
### TABLE 3.2: Genes identified via DLDA as discriminating between PA- and PAMN-treated cells.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Reference Gene ID</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxa3</td>
<td>NM_012823</td>
<td>Annexin A3</td>
</tr>
<tr>
<td>Gstm3</td>
<td>NM_020540</td>
<td>Glutathione S-transferase M3</td>
</tr>
<tr>
<td>Pla2g1b</td>
<td>NM_031585</td>
<td>Phospholipase A2, group IB</td>
</tr>
<tr>
<td>Rab33a</td>
<td>NM_001108257</td>
<td>Member of RAS oncogene family</td>
</tr>
<tr>
<td>RGD1560824_predicted</td>
<td>XR_007343</td>
<td>PREDICTED: Rattus norvegicus similar to Phospholipase A2 precursor</td>
</tr>
<tr>
<td>Serpina5</td>
<td>NM_022957</td>
<td>Serine peptidase inhibitor A5</td>
</tr>
<tr>
<td>Slc2a2</td>
<td>NM_012879</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 2 (GLUT2)</td>
</tr>
<tr>
<td>Snn</td>
<td>NM_001034083</td>
<td>Stannin</td>
</tr>
<tr>
<td>AA998612</td>
<td>TC590201</td>
<td>Rattus norvegicus cDNA clone</td>
</tr>
<tr>
<td>DY472907</td>
<td>FQ217834</td>
<td>RVL24274 Wackym-Soares normalized rat vestibular cDNA library Rattus norvegicus cDNA 5'</td>
</tr>
<tr>
<td>TC603506</td>
<td>TC603506</td>
<td>Q4TA41_TETNG (Q4TA41) Chromosome undetermined SCAF7452, whole genome shotgun sequence. (Fragment), partial (4%)</td>
</tr>
</tbody>
</table>

Anxa3, annexin a3/LC3, lipocortin 3; Gstm3, glutathione S-transferase mu 3; Pla2g1b, phospholipase A2, group IB; Rab33a, RAB33A, member RAS oncogene family; Serpina5, serpin peptidase inhibitor, clade A, member 5/PCI, protein c inhibitor; Slc2a2, solute carrier family 2 (facilitated glucose transporter), member 2/GLUT2, Glucose transporter 2; Snn, stannin.
Following the removal of duplicate genes and genes that were non-homologous to any currently identified *Rattus norvegicus* genes (as described above), the 7 remaining genes were *Rab33a, Serpina5, Anxa3, Pla2g1b, Slc2a2* (GLUT2), *Snn* and *Gstm3*. The relative expression levels for each of these genes as determined in the microarray dataset are shown in Fig. 3.3.

**FIGURE 3.3: Candidate gene expression levels as measured via microarray analysis.**

Relative gene expression levels as measured via microarray analysis between vehicle- (black), PA- (grey) and PAMN- (white) treated cells for the seven genes identified via DLDA. The expression levels of all seven genes were altered with PA-treatment, while PAMN-treatment reversed this change. Data are expressed as mean ± SEM, n=8 for vehicle, n=20 for PA and PAMN, *p*<0.05 versus vehicle-treated, †*p*<0.05 versus PA-treated gene expression levels.

Out of the seven genes, the mRNA levels of five genes were decreased with PA-treatment and restored, or in some instances further enhanced compared with vehicle-treated cells, by PAMN-treatment. *Serpina5* is a serine protease inhibitor that can inactivate multiple anti-coagulant and fibrinolytic enzymes [213]. *Serpina5*
gene expression levels were decreased with PA-treatment by -41 ± 5% \((p=0.0054)\) compared with vehicle, and increased with PAMN-treatment by +15 ± 4% \((p=0.0226)\) compared with PA-treated, but remained lower than vehicle-treated cells \((p=0.0477)\). \textit{Pla2g1b} catalyses the breakdown of glycero-3-phosphocholines to individual fatty acids, and \textit{Pla2g1b} inactivation has been shown to be protective against diet-induced obesity, hyperglycaemia and hyperlipidaemia in mice \cite{214}. \textit{Pla2g1b} gene expression levels were decreased with PA-treatment by -14 ± 2% \((p=0.0142)\) compared with vehicle, and this change in expression was reversed with PAMN-treatment by +33 ± 2% \((p=1.1\times10^{-9})\) compared with PA-treated, which elevated gene expression levels higher than vehicle-treated cells \((p=0.0026)\).

\textit{Slc2a2}, also known as \textit{GLUT2}, is a low affinity glucose transport protein enriched in the liver and kidney \cite{215}. \textit{Slc2a2} mRNA levels were decreased by -19 ± 2% by PA \((p=0.0149)\) compared with vehicle, and increased by +17 ± 3% following PAMN-treatment \((p=0.0035)\) compared with PA-treated. Treatment with PAMN returned \textit{Slc2a2} mRNA levels to the same level as measured in vehicle-treated cells. \textit{Snn} is a transmembrane protein that binds trimethyltin and activates apoptotic cascades, and has been demonstrated to mediate tissue specific sensitivity to organotins \cite{216}. \textit{Snn} mRNA levels following PA-treatment were decreased by -28 ± 5% \((p=0.0206)\) compared with vehicle, and following PAMN-treatment increased by +23 ± 5% \((p=0.0022)\), returning to the same level as in vehicle-treated cells. \textit{Gstm3} is a cytoplasmic glutathione-s-transferase subunit, which interacts with glutathione to detoxify numerous toxins, and has been shown to be decreased in rodent models of hyperglycaemia \cite{217}. \textit{Gstm3} gene expression levels following PA-treatment were decreased by -29 ± 5% \((p=0.0159)\) compared with vehicle, and following PAMN-
Two out of the potential seven GES genes increased with PA-treatment and in turn were decreased following PAMN-treatment. \textit{Rab33a} is a member of the small GTP-binding protein family which play a role in intracellular vesicular transport [218]. The mRNA levels of \textit{Rab33a} increased by $+18 \pm 4\%$ with PA-treatment ($p=0.0413$ compared with vehicle), while following PAMN-treatment, \textit{Rab33a} decreased by $-33 \pm 4\%$ ($p=1.0\times10^{-6}$ compared with PA-treated) to similar levels as measured in the vehicle-treated cells. \textit{Anxa3} is a member of a calcium-dependant phospholipid-binding protein family, which inhibits the action of phospholipase A2 family members [219]. \textit{Anxa3} mRNA levels increased with PA-treatment by $+91 \pm 14\%$ ($p=0.0099$ compared with vehicle). Following PAMN-treatment, \textit{Anxa3} expression decreased by $-48 \pm 12\%$ ($p=0.0115$ compared with PA-treated), restoring gene expression to the same levels as measured in the vehicle-treated cells.

\textbf{3.3.2. Validation of the potential PA-based GES genes using RT-PCR}

The potential GES genes were validated using RT-PCR in technical replicates to confirm the microarray results with an alternative technology, as well as in biological replicates to establish the reproducibility of the microarray results (Fig. 3.4). Of the genes that decreased with PA-treatment in the microarray data, and were increased (restored) with PAMN-treatment (\textit{Serpina5, Pla2g1b, Slc2a2, Snn} and \textit{Gstm3}), none were validated in either the technical replicates or biological replicates via RT-PCR analysis (Fig. 3.4 a). PA-treatment did not significantly alter the
expression levels of any of these genes. The only genes to show a similar expression profile to the microarray data were *Serpina5* and *Pla2g1b*, however these changes were not significant across both validation experiments. In contrast to the microarray data, the gene expression of *Gstm3* following PAMN-treatment was decreased (-55 ± 9%, \( p=0.0331 \)) compared with PA-treated cells (Fig. 3.4 b).

Similarly, of the genes which were increased with PA-treatment and lowered (restored) with PAMN-treatment (*Rab33a* and *Anxa3*), neither were validated in either the technical or biological replicate samples. *Rab33a* gene expression changed in direct contrast to the observed trends measured in the microarray data, while the expression changes to *Anxa3* in response to PA-treatment were only observed in the technical replicates and were not reproduced in the biological replicate samples (Fig. 3.4 a-b).

In summary, the gene expression levels of *Rab33a* and *Gstm3* in both RT-PCR validation experiments were opposite to the gene expression levels observed in the original microarray data, and as such they were not carried forward for further experimentation. The gene expression levels of *Slc2a2* and *Snn* were not significantly altered in the validation experiments in response to either PA- or PAMN-treatment. Therefore, *Slc2a2* and *Snn* were not validated, and not carried forward for further experimentation. The trends for gene expression changes for *Serpina5*, *Anxa3* and *Pla2g1b* were consistent with the changes observed in the original microarray data, however one or more of the statistical differences observed in the original analysis were not replicated by RT-PCR. This may have been due to the larger sample size in the original microarray experiments (\( n= 8 \) for
vehicle, and n=20 for PA and PAMN) compared with the validation experiments (n=4 for the microarray samples, n=6 for the biological replicate samples). In the absence of genes with full validation in both the technical and biological replicates, the generation of a GES in this model was unsuccessful using DLDA. Further analysis of the microarray dataset is currently ongoing using a Bayesian modelling approach to identify an increased number of candidate genes to be validated. This modelling has been recently completed by our collaborators (Professor John Blangero, Dr. Tom Dyer, Dr Marcio Almeida and Dr. Joanne Curran at Texas Biomedical Research Institute, San Antonio), and validation of these genes is a future direction for this study.
FIGURE 3.4: Candidate GES gene expression levels as measured via RT-PCR.
Relative gene expression levels measured via RT-PCR of either a random subset of samples used for microarray analysis (technical replicates) (a) or a separate set of biological replicate samples (b). Gene expression levels shown between vehicle-(black), PA- (grey) and PAMN- (white) treated cells for the seven genes identified via DLDA. Data are expressed as mean ± SEM, (a) n=4 or (b) n=6, *p<0.05 versus vehicle gene expression levels, †p<0.05 versus PA-treated gene expression levels.
3.3.3. Human phenotypic profiling of the candidate gene Anxa3

In the absence of any fully validated genes from the GES candidates identified through DLDA, the genes Serpina5, Anxa3 and Pla2g1b were considered as potential candidates for phenotype analysis in a human dataset, as they showed similar trends for gene expression between RT-PCR and microarray data, however this was not consistently different in all data sets. Data from the San Antonio Family Heart Study (SAFHS, [150]) was mined for each of the three candidate genes Serpina5, Anxa3 and Pla2g1b as proof of principle to determine whether any correlations between human gene expression data and a series of phenotypes relevant to the metabolic syndrome could be determined. Of the three candidate genes, only Anxa3 was detected in the human lymphocyte expression data from the SAFHS. The correlation data is summarised in Table 3.3 below.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Correlation Coefficient (r)</th>
<th>Significance (2-tailed)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOMA-β</td>
<td>-0.008</td>
<td>0.791</td>
<td>1223</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>-0.015</td>
<td>0.603</td>
<td>1223</td>
</tr>
<tr>
<td>Total Serum Cholesterol (mM)</td>
<td>0.035</td>
<td>0.225</td>
<td>1237</td>
</tr>
<tr>
<td>HDL Cholesterol (mM)</td>
<td>-0.020</td>
<td>0.492</td>
<td>1236</td>
</tr>
<tr>
<td>Total Serum Triglyceride</td>
<td>0.069</td>
<td>0.015*</td>
<td>1237</td>
</tr>
<tr>
<td>Fasting Glucose (mM)</td>
<td>-0.066</td>
<td>0.033*</td>
<td>1051</td>
</tr>
<tr>
<td>Fasting Insulin</td>
<td>-0.007</td>
<td>0.818</td>
<td>1035</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>0.026</td>
<td>0.368</td>
<td>1224</td>
</tr>
<tr>
<td>Waist Circumference</td>
<td>-0.002</td>
<td>0.950</td>
<td>706</td>
</tr>
</tbody>
</table>

Anxa3, annexin a3/LC3, lipocortin 3; HOMA-β, homeostatic model assessment – beta cell function; HOMA-IR, homeostatic model assessment – insulin resistance; HDL, high-density lipoprotein; BMI, body mass index. *p<0.05.
No correlation was found between Anxa3 gene expression levels and either of the HOMA scores for insulin and glucose homeostasis, HOMA-β and HOMA-IR. Related to this, there was no correlation between Anxa3 mRNA levels and fasting circulating insulin levels. However, there was a significant negative correlation (-0.066, \( p=0.033 \), Table 3.3) between fasting plasma glucose levels and Anxa3 mRNA levels. There was also a positive correlation (0.069, \( p=0.015 \), Table 5.3) between total serum triglyceride and Anxa3 gene expression levels. This correlation is in agreement with the significant increase in triglyceride levels observed in PA-treated FAO cells (Chapter 5). Linear regression analysis was performed, and when adjusted for age, sex and BMI, Anxa3 was not an independent predictor of either fasting plasma glucose or total serum triglyceride (data not shown).
3.4. Discussion

The initial aim of this chapter was the collection of a global gene expression profile for PA-induced insulin resistance and dysregulation of glucose production, alongside the reversal of the high glucose production with PAMN treatment. There were 16,276 genes that passed quality control measures following normalisation of the data. The gene expression data will be used for further analysis in Chapter 4 (of regulated signalling and metabolic gene networks). Therefore the first aim of this chapter has been successfully completed - the measurement of whole genome transcriptional changes between vehicle, PA and PAMN-treated cells.

While many studies have investigated *in vitro* and *in vivo* gene expression in response to elevated fatty acids or obesity (see Chapter 4 for further discussion), our laboratory is unaware of any studies using this data for the generation of a GES as performed in this study. Unfortunately, the putative GES genes identified in this study were unable to be validated *in vitro*, and as such the identity of a PA-based hepatic GES remains unresolved at this stage.

Due to the identification of only 14 genes via DLDA, and the inability to validate any of those gene expression changes in the technical and biological replicate samples, the secondary aim of this chapter to identify a GES has not been successfully completed. Despite this outcome, there remains significant utility for the microarray dataset detailed in this thesis for future study. Firstly, the use of Bayesian statistical modelling to compare the discriminating power of all possible gene combinations from the microarray data [147] will lead to the identification of a greater number of candidate GES genes. This approach will therefore maximise the
Chapter 3: Identification of candidate GES genes

chances of identification of a subset of GES genes that are able to be validated in both the technical and biological replicate samples, as well as being expressed in human lymphocytes (analysis currently in progress). This approach has been utilised previously in the successful development of a TNFα-induced insulin resistance GES by our laboratory [141]. In this previous study, Bayesian modelling identified 20 different GESs, comprising a total of 20 genes. The low validation rate described in this chapter may be due to a number of factors. Numerous studies have reported significant variation between microarray and RT-PCR data when the measured gene expression changes are less than 2-fold [220-222], as was the case for all of the microarray data in this study. Studies have also found that increased distance between the PCR primer location and microarray probe location lead to a reduction in correlation between the two data sets [221]. Therefore, designing RT-PCR primers closer to the microarray oligonucleotide sequences may improve the correlation between the two techniques. A number of studies have also noted higher correlation between microarray and RT-PCR datasets for up-regulated genes, as a consequence of increased variability in the lower-intensity array spots for down-regulated genes [223, 224]. Given that five of the seven candidate genes in this study were down-regulated with PA-treatment, this may in part explain the poor correlation between the microarray and RT-PCR data.

As an alternative to the gene expression based signatures, lipidomic, metabolomic and proteomic-based approaches have been successfully employed for other diseases. A metabolic signature for Alzheimer’s disease has been developed in human plasma from a subset of known signalling proteins [225]. One recent study has described plasma amino acid based signatures which alter with both age and
progression of a prediabetic state in dominant-negative glucose-dependant insulinotropic polypeptide receptor (GIPR\textsuperscript{dn}) transgenic pigs \[226\]. The same study also profiled plasma lipids and described altered plasma concentrations for a number of lipid species which correlated with glucose tolerance, insulin secretion and total β-cell volume \[226\]. Given that the lipidomic profiling of FAO hepatoma cells has already been completed in this study (see \textbf{Chapter 5}), it would be possible to generate a lipid-based signature with the current data. This approach poses increased technical challenges in patient or compound screening due to the increased complexity for sample preparation and analysis. Despite this limitation, future technical advances in the measurement of these metabolites via mass spectroscopy or alternative technologies such as nuclear magnetic resonance (NMR) spectroscopy may make this approach viable.

The first aim of this chapter – to utilise microarray analysis to identify the whole genome expression profile of FAO hepatoma cells in response to PA and PAMN treatment was successfully completed, and this dataset will be further utilised in \textbf{Chapter 4}. The second aim of \textbf{Chapter 3} – the identification and validation of putative GES genes was only partially completed. Seven candidate GES genes were identified using DLDA, however none of the putative GES genes were validated in the technical or biological replicates. As a consequence of this, the tertiary aim of the chapter was completed as proof of principle, using the one gene (\textit{Anxa3}) which followed the same trends in the technical and biological replicate data and was present in the SAFHS human lymphocyte dataset. The gene expression of Anxa3 was confirmed in the human lymphocytes, and found to be negatively correlated with fasting glucose, however this was not an independent association when
adjusted for age, sex or BMI. Neither BMI or waist circumference were found to be correlated with Anxa3 expression, an interesting observation given that the gene was identified in a model of hyperlipidemia-induced glucose dysregulation.

Given the changes in gene expression levels associated with metabolism of cholesterol discussed in Chapter 4, it was of particular interest to determine whether Anxa3 gene expression was correlated with both total serum cholesterol and HDL-cholesterol. However, there was no significant correlation between Anxa3 gene expression and either total serum cholesterol or HDL-cholesterol in the SAFHS cohort. A weak positive correlation between total serum triglyceride and Anxa3 gene expression was found, however this association was not independent of age, sex and BMI.

The role of Anxa3 gene expression in insulin resistance and obesity can be established via its interaction with another of the candidate genes – Pla2g1b. Anxa3 has been shown to inhibit the activity of phospholipase proteins [219]. Phospholipases, specifically Pla2g1b, have been implicated in obesity-related insulin resistance in numerous animal models. In addition, studies in human patients have shown evidence of an association between SNPs in PLA2G1B and central fat mass [227]. Pla2g1b−/− mice, have been shown to have lower postprandial glycaemia compared with wild type controls. This is due to increased postprandial glucose uptake in the liver, heart and muscle tissues. It was shown that Pla2g1b-mediated lysophospholipid absorption contributes to this postprandial hyperglycaemia [228], as intraperitoneal injections of lysophospholipids ablated the increased glucose uptake effects observed in Pla2g1b−/− mice. Lysophospholipid species have been
shown to suppress hepatic fatty acid oxidation [229], and have been shown to suppress insulin-stimulated hepatic glycogen synthesis \textit{in vitro}. In contrast, $\text{Pla2g1b}^{-/-}$ mice exhibit increased postprandial hepatic fat oxidation, through increased gene expression levels of $\text{PPAR}\alpha$, $\text{PPAR}\delta$, $\text{PPAR}\gamma$ and mitochondrial uncoupling protein 2 ($\text{Ucp2}$) [229]. Protection against diet-induced hyperlipidemia [214], obesity and obesity-driven insulin resistance [230] in $\text{Pla2g1b}^{-/-}$ mice have all been described. It is therefore the effects of Pla2g1b on downstream control of hepatic fatty acid oxidation that are responsible for its effects on glucose metabolism in these mice.

Further, Pla2g1b has been successfully pharmacologically targeted. High-fat fed mice treated with the Pla2g1b inhibitor methyl indoxam were protected from developing diet-induced obesity and glucose intolerance, and this was correlated with a decrease in postprandial lysophospholipid absorption [231]. The effects of Pla2g1b appear to be closely related to its role in the digestive tract, as overexpression of $\text{Pla2g1b}$ in pancreatic acinar cells has been shown to exacerbate diet-induced obesity and insulin resistance in rodent models [232]. Therefore, it may be that reducing Pla2g1b activity proximal to the digestive tract is a viable mediator for its role in lysophospholipid absorption.

In summary, the development of a PA-based GES was unsuccessful in this study. However, despite a non-biased approach to find candidate genes, two mediators of hepatic lipid oxidation and glucose metabolism were identified ($\text{Anxa3}$ and $\text{Pla2g1b}$) proved the value of this approach. It is therefore possible that with further study this approach could be successful in identifying additional novel mediators of glucose metabolism.
Chapter 4 – Global gene expression profiling of PA-induced dysregulation of hepatic glucose production, and restoration with metformin and sodium salicylate

4.1. Introduction

Gene set enrichment analysis (GSEA) is a computational software package which can be used to determine whether specific gene sets (grouped on functional similarity), are overrepresented in a given set of differentially expressed genes between two biological states or tissues. GSEA can therefore be used to indicate whether a correlation exists between differential expression of a specific set of genes (pathway), and the phenotype being investigated [127, 128]. The main advantage of the GSEA approach is that it simplifies often highly complex data sets into more biologically relevant information [127]. By grouping genes according to their biological function, gene expression changes which may appear minimal at the level of a single gene can become significant when viewed as part of the wider regulation of a larger signalling or metabolic network. Further, it is now becoming apparent that another advantage of the GSEA approach to classifying disease includes improved reproducibility across samples sets compared with individual markers of disease [233]. This approach has been successfully applied to the classification of tumour subtypes, and therefore has the potential to accurately guide treatment selection compared with single marker identification.
There have been relatively few studies investigating changes to whole genome expression in the face of hyperlipidaemia-induced dysregulation of glucose production in the liver [234, 235], and there are no published studies to date reporting on both the induction and reversal of such a state. This study will therefore provide a novel insight into the transcriptional changes accompanying dysregulated glucose production, as well as successful treatment of such a phenotype. Therefore, the aim of this chapter was to perform GSEA on the microarray data collected in Chapter 3 to determine the regulation of metabolic and signalling pathways at the whole genome level.
4.2. Methods

![Flowchart for identification of regulated pathway using DAVID.](image)

**FIGURE 4.1: Flowchart for identification of regulated pathway using DAVID.**
Flowchart of methodology from collection of RNA samples for microarrays, through to data analysis and GSEA using Database for Annotation, Visualization and Integrated Discovery (DAVID) software package.

4.2.1. Gene Set Enrichment Analysis (GSEA) using DAVID

Selection criteria for samples to undergo microarray analysis, RNA extraction and microarray analysis was performed as described in Chapter 3. Paired t-tests were used to identify genes, from the normalised microarray data, with evidence of differential expression between vehicle- and PA-treated cells (450 genes with nominal $p<0.05$), as well as between PA- and PAMN-treated cells (1434 genes with...
Chapter 4: Gene set enrichment analysis

nominal \( p<0.05 \)). The complete gene lists of putative differentially expressed genes were then imported into the online Database for Annotation, Visualization and Integrated Discovery (DAVID) software package v6.7 [236, 237]. Using the functional annotation tools, the data was analysed to determine enrichment values for defined Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways. Pathways were selected based on significant enrichment using Fisher exact statistical analysis \( (p<0.05) \). Pathway data was expressed as a percentage change from vehicle- to PA-treated cells, or from PA- to PAMN-treated cells. All data was expressed as mean ± SEM.
4.3. Results

4.3.1. Gene pathways induced with PA-mediated dysregulation of glucose production

Microarray analysis identified 450 genes with evidence for differential expression between vehicle- and PA-treated cells (nominal $p<0.05$). This set of 450 genes were assessed by DAVID to provide an unbiased approach to rank the global gene expression data into pathways of biological significance by identifying groups of genes that are enriched and functionally related. The GSEA analysis of vehicle-versus PA-treated cells identified 17 pathways (nominal $p<0.05$) involved in a range of processes including cell cycle regulation, cancer progression, and inositol phosphate metabolism (Table 4.1). All gene expression levels in this section are reported as the percentage change in PA-treated cells compared with vehicle-treated cells. All pathways identified as being differentially expressed between vehicle- and PA-treated cells are represented alongside relevant changes in gene expression in Appendix 4.
### TABLE 4.1: Pathways identified as significantly overrepresented in the list of differentially expressed genes between vehicle- and PA-treated cells.

<table>
<thead>
<tr>
<th>Defined KEGG Pathway</th>
<th>Number of differentially expressed genes in pathway</th>
<th>Fold Enrichment</th>
<th>Fisher Exact p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell cycle</td>
<td>8 / 126</td>
<td>4.9</td>
<td>1.9E-4</td>
</tr>
<tr>
<td>Oocyte meiosis</td>
<td>6 / 111</td>
<td>4.2</td>
<td>2.9E-3</td>
</tr>
<tr>
<td>Toll-like receptor signalling pathway</td>
<td>5 / 90</td>
<td>4.3</td>
<td>5.8E-3</td>
</tr>
<tr>
<td>Renal cell carcinoma</td>
<td>4 / 69</td>
<td>4.5</td>
<td>1.2E-2</td>
</tr>
<tr>
<td>Leukocyte transendothelial migration</td>
<td>5 / 115</td>
<td>3.4</td>
<td>1.6E-2</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>4 / 81</td>
<td>3.8</td>
<td>2.0E-2</td>
</tr>
<tr>
<td>Neutrophin signalling pathway</td>
<td>5 / 126</td>
<td>3.1</td>
<td>2.3E-2</td>
</tr>
<tr>
<td>Progesterone-mediated oocyte maturation</td>
<td>4 / 87</td>
<td>3.6</td>
<td>2.5E-2</td>
</tr>
<tr>
<td>Antigen processing and presentation</td>
<td>4 / 88</td>
<td>3.5</td>
<td>2.6E-2</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>4 / 90</td>
<td>3.5</td>
<td>2.8E-2</td>
</tr>
<tr>
<td>Inositol phosphate metabolism</td>
<td>3 / 52</td>
<td>4.5</td>
<td>2.9E-2</td>
</tr>
<tr>
<td>Melanogenesis</td>
<td>4 / 92</td>
<td>3.4</td>
<td>3.0E-2</td>
</tr>
<tr>
<td>GnRH signalling pathway</td>
<td>4 / 94</td>
<td>3.3</td>
<td>3.2E-2</td>
</tr>
<tr>
<td>RNA degradation</td>
<td>3 / 57</td>
<td>4.1</td>
<td>3.7E-2</td>
</tr>
<tr>
<td>Wnt signalling pathway</td>
<td>5 / 145</td>
<td>2.7</td>
<td>3.8E-2</td>
</tr>
<tr>
<td>Focal adhesion</td>
<td>6 / 195</td>
<td>2.4</td>
<td>3.9E-2</td>
</tr>
<tr>
<td>NOD-like receptor signalling pathway</td>
<td>3 / 62</td>
<td>3.8</td>
<td>4.5E-2</td>
</tr>
</tbody>
</table>

Number of differentially expressed genes in pathway expressed as: number of differentially expressed genes from KEGG pathway identified in array data/total number of genes in defined KEGG pathway; Fold enrichment quantifies the overrepresentation of a specific KEGG pathway in the differentially expressed genes identified in microarray data. GnRH, gonadotropin-releasing hormone; NOD, Nucleotide-oligomerization domain. These pathways are represented along with changes in gene expression in Appendix 4.
4.3.1.1. Gene set enrichment analysis between vehicle- and PA-treated FAO cells identified multiple regulatory pathways in cell cycle regulation

Common to nine of the 17 pathways regulated with PA-treatment is the theme of cell cycle, proliferation and cancer progression. Pathways involved in cell cycle regulation include cell cycle, oocyte meiosis, neutrophin signalling pathway, progesterone mediated oocyte maturation, Wnt signalling pathway and focal adhesion. Overall, there was a consistent down-regulation of genes involved in the progression of the cell cycle pathway (see Appendix 4).

In addition to direct control of the cell cycle, growth and proliferative signalling was a common theme amongst the above pathways. The neutrophin signalling pathway, focal adhesion pathway, toll-like receptor signalling pathway, renal cell carcinoma, leukocyte transendothelial migration, colorectal cancer and prostate cancer all contained a central signalling network including members of proliferative (and insulin-induced) signalling cascades such as phosphoinositide-3-kinase regulatory subunit 3 gamma (Pik3r3, -35 ± 3%, nominal \(p=0.0382\)) and its downstream target catenin beta 1 (Ctnnb1, -21 ± 5%, nominal \(p=0.0404\)) which together regulate cell growth, and cellular adhesion through anchoring the cytoskeleton [238, 239].

The largest increase in gene expression common to these pathways were both Fos (+124 ± 52%, nominal \(p=0.0389\)) and Jun (+119 ± 27%, nominal \(p=0.0007\)), downstream of the stress kinase JNK, which form the transcription complex AP-1 – a regulator of both cell cycle and apoptotic signalling genes. The decrease in Jun expression was also common to the gonadotropin-releasing hormone signalling
pathway, which contains genes regulating the synthesis and release of glycoprotein hormones from the pituitary, as well as the toll-like receptor signalling pathway.

In summary, both the cell cycle and cancer pathways were negatively regulated in their control of normal cell proliferative mechanisms following PA-treatment, and positively regulated in their control of apoptotic signalling. This may in part explain the decrease observed in cellular viability and the increased cellular cytotoxicity (see Chapter 2) observed with PA-treatment. The negative regulation of normal cell proliferative signalling along with the increased apoptotic signalling support this conclusion, however as neither of these factors have been directly measured it is currently unclear whether this was due to decreased proliferation or increased apoptosis, or a combination of the two factors.

4.3.1.2. Gene set enrichment analysis between vehicle and PA-treated FAO cells identified multiple regulatory pathways in immune function and inflammation

Four pathways with defined roles in immune function and inflammation were identified as being significantly regulated following PA-treatment - the toll-like receptor signalling pathway, leukocyte transendothelial migration, antigen processing and presentation and nucleotide-oligomerization domain (NOD)-like receptor signalling pathway. Common to these pathways are inducers of inflammatory cytokines including signalling members Fos and Jun (see above) as well as inflammasome mediators cytosolic heat shock protein of 90 kDa alpha class B, member 1 (Hspcb, -17 ± 4%, nominal \( p=0.0484 \)), and Erbb2 interacting protein
(Ebin, -30 ± 4%, nominal $p=0.0361$), which is upstream of a number of mediators of apoptosis (including Caspase 8) and proinflammatory cytokine production (JNK and NF-kB). The combined data from these pathways indicates an inflammatory response to PA-treatment in these cells.

4.3.1.3. Gene set enrichment analysis between vehicle and PA-treated FAO cells identified regulation of inositol phosphate metabolic pathways

Due to their roles as key molecules in the insulin signal transduction pathway, regulation of phosphatidylinositols through the inositol phosphate metabolism pathway is of specific interest and relevance to the observed dysregulation of the insulin endpoint action of glucose production in this model. The mRNA levels of the PIP$_3$ generating inositolphosphate multikinase ($Ipmk$ [240]) was decreased with PA-treatment (-29 ± 5%, nominal $p=0.0197$). Additionally the gene expression levels of the enzyme responsible for the removal of 1,2-diacylglycerols from the inositol phosphate metabolic pathway ($Plcb1$) were also decreased (-32 ± 5%, nominal $p=0.0273$). Given the importance of phosphatidylinositols in signal transduction in the insulin signalling cascade, the identification of the regulation of this pathway by PA-treatment was significant and warrants further investigation (see Chapter 5).
4.3.2. Regulated gene pathways induced with PAMN-mediated reversal of PA-induced dysregulation of glucose production

Microarray analysis identified 1434 genes as being differentially expressed between PA- and PAMN-treated cells (nominal $p<0.05$). GSEA of the microarray data resulted in the identification of 24 pathways (nominal $p<0.05$) that were reported as significantly regulated by PAMN-treatment according to DAVID analysis (Table 4.2). The pathways involved biological processes such as fatty acid synthesis and metabolism, sphingolipid metabolism, PPAR signalling and glutathione metabolism. In contrast to the pathways identified above by DAVID as being significantly regulated when comparing vehicle- with PA-treated cells, no identical pathways were identified when comparing PA- with PAMN-treated cells. Some similar functions were identified however, with pathway regulation of immune response and mediators of inflammation occurring in both GSEA comparisons (see below). This largely disparate transcriptional response implies mostly different mechanisms for the induction of an insulin resistant, increased glucose production phenotype with PA-treatment compared with the lower glucose production phenotype observed with PAMN-treatment. All pathways identified as being differentially expressed between PA- and PAMN-treated cells are represented alongside relevant changes in gene expression in Appendix 5.
**TABLE 4.2: Pathways identified as significantly overrepresented in the list of differentially expressed genes between PA- and PAMN-treated cells.**

<table>
<thead>
<tr>
<th>Defined KEGG Pathway</th>
<th>Number of differentially expressed genes in pathway</th>
<th>Fold Enrichment</th>
<th>Fisher Exact p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid biosynthesis</td>
<td>11 / 17</td>
<td>12.6</td>
<td>4.9E-11</td>
</tr>
<tr>
<td>Terpenoid backbone biosynthesis</td>
<td>9 / 14</td>
<td>12.6</td>
<td>3.4E-9</td>
</tr>
<tr>
<td>Metabolism of xenobiotics by CYT. P450</td>
<td>16 / 60</td>
<td>5.2</td>
<td>2.8E-8</td>
</tr>
<tr>
<td>Drug metabolism – CYT. p450</td>
<td>17 / 72</td>
<td>4.6</td>
<td>7.4E-8</td>
</tr>
<tr>
<td>PPAR signalling pathway</td>
<td>16 / 71</td>
<td>4.4</td>
<td>3.6E-7</td>
</tr>
<tr>
<td>Complement and coagulation cascades</td>
<td>15 / 70</td>
<td>4.2</td>
<td>1.7E-6</td>
</tr>
<tr>
<td>Porphyrin and chlorophyll metabolism</td>
<td>8 / 31</td>
<td>5.0</td>
<td>1.2E-4</td>
</tr>
<tr>
<td>Biosynthesis of unsaturated fatty acids</td>
<td>7 / 24</td>
<td>5.7</td>
<td>1.4E-4</td>
</tr>
<tr>
<td>Steroid hormone biosynthesis</td>
<td>9 / 43</td>
<td>4.1</td>
<td>2.6E-4</td>
</tr>
<tr>
<td>Retinol metabolism</td>
<td>10 / 59</td>
<td>3.3</td>
<td>7.1E-4</td>
</tr>
<tr>
<td>Drug Metabolism – Other Enzymes</td>
<td>8 / 44</td>
<td>3.6</td>
<td>1.5E-3</td>
</tr>
<tr>
<td>Arachidonic acid metabolism</td>
<td>10 / 70</td>
<td>2.8</td>
<td>2.7E-3</td>
</tr>
<tr>
<td>Glutathione metabolism</td>
<td>8 / 50</td>
<td>3.1</td>
<td>3.5E-3</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>11 / 90</td>
<td>2.4</td>
<td>5.8E-3</td>
</tr>
<tr>
<td>Butanoate metabolism</td>
<td>6 / 33</td>
<td>3.6</td>
<td>5.8E-3</td>
</tr>
<tr>
<td>Synthesis and degradation of ketone bodies</td>
<td>3 / 9</td>
<td>6.5</td>
<td>8.8E-3</td>
</tr>
<tr>
<td>Fatty acid metabolism</td>
<td>6 / 42</td>
<td>2.8</td>
<td>1.9E-2</td>
</tr>
<tr>
<td>Sphingolipid metabolism</td>
<td>6 / 42</td>
<td>2.8</td>
<td>1.9E-2</td>
</tr>
<tr>
<td>Linoleic acid metabolism</td>
<td>5 / 33</td>
<td>3.0</td>
<td>2.5E-2</td>
</tr>
<tr>
<td>Nicotinate and nicotinamide metabolism</td>
<td>4 / 23</td>
<td>3.4</td>
<td>2.8E-2</td>
</tr>
<tr>
<td>Valine, leucine, and isoleucine degradation</td>
<td>6 / 46</td>
<td>2.5</td>
<td>2.8E-2</td>
</tr>
<tr>
<td>RIG-1-like receptor signalling pathway</td>
<td>7 / 60</td>
<td>2.3</td>
<td>3.2E-2</td>
</tr>
<tr>
<td>Pentose and glucuronate interconversions</td>
<td>3 / 15</td>
<td>3.9</td>
<td>3.8E-2</td>
</tr>
<tr>
<td>Primary bile acid synthesis</td>
<td>3 / 15</td>
<td>3.9</td>
<td>3.8E-2</td>
</tr>
</tbody>
</table>

Number of differentially expressed genes in pathway expressed as: number of differentially expressed genes from KEGG pathway identified in array data/total number of genes in defined KEGG pathway; Fold enrichment quantifies the overrepresentation of a specific KEGG pathway in the differentially expressed genes identified in microarray data. CYT., cytochrome. These pathways are represented along with changes in gene expression in Appendix 5.
4.3.2.1. **Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified multiple regulatory pathways of both the fatty acid and amino acid biosynthetic pathways**

The GSEA analysis identified 5 pathways regulating fatty acid and amino acid biosynthesis, which all produce acetyl-CoA as an intermediary: biosynthesis of unsaturated fatty acids, fatty acid metabolism, synthesis and degradation of ketone bodies, butanoate metabolism and valine, leucine and isoleucine degradation (Fig. 4.2).

In the biosynthesis of unsaturated fatty acids pathway, short and medium chain fatty acid elongase (Elov16, -19 ± 6%, nominal \( p=0.0265 \)) and desaturase (Fads2, -25 ± 6%, nominal \( p=0.0239 \)) were decreased in PAMN-treated cells compared with PA-treated cells, alongside increased acyl-CoA acyltransferase 1 (Acaa1, +15 ± 5%, nominal \( p=0.0356 \)) and thioesterase (Acot1, Acot2, Acot4, and Acot5) enzymes. These changes indicate a shift away from medium chain fatty acid metabolism and an increased ability to generate acetyl-CoA and free fatty acids from fatty acyl-CoA molecules.

In the fatty acid metabolism pathway, the very long chain acyl-CoA dehydrogenase (Acadvl) was decreased with PAMN-treatment (-21 ± 4%, nominal \( p=0.0078 \)), however there was an increase to enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase (Ehhadh, +108 ± 24%, nominal \( p=0.0004 \)) as well as hydroxyacyl-CoA dehydrogenase short chain (Hadhsc, +9 ± 3%, nominal \( p=0.036 \)). The increased expression levels of these genes may be an indicator of increased conversion of medium and short chain fatty acids to acetyl-CoA.
The synthesis and degradation of ketone bodies pathway also regulates metabolism of acetoacetate and acetyl-CoA. The genes acetyl-CoA acyltransferase 2 (Acat2, -37 ± 4%, nominal $p=8.8\times10^{-9}$), the soluble 3-hydroxy-3-methylglutaryl-CoA synthase 1 (Hmgcs1, -23 ± 4%, nominal $p=0.0055$) and mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase 2 (Hmgcs2, -27 ± 6%, nominal $p=0.0193$) were all decreased in this pathway. These genes all metabolise acetyl-CoA into either acetoacetyl-CoA or acetoacetate, therefore, the observed decrease in mRNA levels may indicate decreased utilisation of acetyl-CoA by this metabolic pathway.

The butanoate metabolism pathway also identified many of the same genes that metabolise acetyl-CoA, including decreased expression of genes that irreversibly metabolise acetyl-CoA (Hmgcs1 and Hmgcs2, as noted above), and both decreased (acetoacetyl-CoA synthetase, Aacs, -22 ± 6%, nominal $p=0.0304$) and increased (Ehhadh and Hadhsc, as noted above) expression of genes feeding into acetyl-CoA production. The direction of acetyl-CoA synthesis is therefore less clear in this pathway. With the exception of Aacs, these same genes were also identified in the branch chain amino-acid metabolism pathway valine, leucine and isoleucine degradation, further highlighting the regulation of metabolic pathways involving acetyl-CoA metabolism following PAMN-treatment. The enzymes leading to acetyl-CoA synthesis in this pathway however were both increased and decreased, with no clear pattern observed. The relationship between these pathways and acetyl-CoA metabolism is summarised in Fig. 4.2.
FIGURE 4.2: PAMN-induced changes in metabolic pathways regulating acetyl-CoA metabolism.
Biosynthesis pathways that include acetyl-CoA as an intermediate were identified as being regulated at the transcriptional level between PA-and PAMN-treated cells via DAVID analysis. The thickness and direction of the arrows indicate the effect the observed gene expression changes would have on the ability of the cell to metabolise acetyl-CoA. The transcriptional regulation observed with PAMN treatment indicates a potential for increased acetyl-CoA production through the fatty acid metabolism and biosynthesis of unsaturated fatty acid pathways, as well as a decreased utilisation of acetyl-CoA by the synthesis and degradation of ketone bodies pathway. These factors combined indicate an increased availability of acetyl-CoA as a metabolite of downstream metabolic pathways such as terpenoid backbone biosynthesis.
4.3.2.2. Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified changes in the cholesterol and bile acid synthetic pathways

There were four key pathways that were induced between PA-and PAMN-treatment which followed directly from acetyl-CoA synthesis (as described above) through to steroid hormone and bile acid production. These pathways follow the conversion of acetyl-CoA to farnesyl-PP (Terpenoid Backbone Biosynthesis pathway, fold enrichment 12.6, \( p=3.4 \times 10^{-9} \), Fig. 4.3 a), farnesyl-PP to cholesterol (Steroid Biosynthesis pathway, fold enrichment 12.6, \( p=4.9 \times 10^{-11} \), Fig. 4.3 b) and Cholesterol to c18-, c19- and c21-steroid hormones (Steroid Hormone Biosynthesis pathway, fold enrichment 4.1, \( p=2.6 \times 10^{-4} \), Fig. 4.3 c) or cholesterol to primary bile acids (Primary bile acid synthesis, fold enrichment 3.9, \( p=3.8 \times 10^{-3} \)). The metabolic link between these pathways is detailed in figure 4.3 d. All 20 enzymes in the metabolic pathway regulating the conversion of acetyl-CoA to cholesterol were reduced in the PAMN-treated group compared with PA-alone treated cells, suggesting a decreased ability of the cells to synthesise cholesterol, and therefore any downstream metabolic products such as steroid hormones or bile acids. These pathways are discussed in further detail below.
FIGURE 4.3: Biosynthesis pathways identified as being regulated at the transcriptional level between PA-and PAMN-treated cells via GSEA.

(a) Downregulation of all nine enzymes of the terpenoid backbone biosynthesis pathway, following the continuous conversion of acetyl-CoA to farnesyl-PP. (b) Downregulation of all 11 enzymes of the steroid biosynthesis pathway, following the continuous conversion of farnesyl-PP to cholesterol. (c) Regulation of 9 enzymes of the steroid hormone biosynthesis pathway, following the conversion of cholesterol to steroid hormones. The direct linkage of these three pathways, as well as primary bile acid biosynthesis, is highlighted in (d). Relative amounts of each transcript reported as percentage change from PA to PAMN and represented as mean ± SEM. All expression changes detailed were significant at nominal p<0.05.
For the first of these linked pathways, Terpenoid backbone biosynthesis, all 9 members showed decreased expression in PAMN-treated cells compared with PA-treated cells (Fig. 4.3 a), including the enzyme mevalonate kinase (Mvk, -19 ± 5%, nominal $p=0.043$). Similarly in the second pathway, Steroid Biosynthesis, all eleven members showed decreased expression in PAMN-treated cells compared with PA-treated cells (Fig. 4.3 b). The third pathway in this group, Steroid Hormone Biosynthesis, did not follow this trend, with both increased and decreased gene expression in PAMN-treatment compared with PA-treated cells (Fig. 4.3 c). Both up and downregulation was observed in the synthetic pathways leading to C21-steroids, as well as the branches of the pathway leading to C18/C19-steroids. These three pathways form a direct and continuous metabolic link from acetyl-CoA to cholesterol, and then diverge to C18-, C19- and C21 steroid hormones (Fig. 4.3 d).

In addition, the associated pathway of Primary Bile Acid Biosynthesis follows the conversion of cholesterol, through to the two primary bile acids cholate and chenodeoxycholate. There was decreased expression in three of the genes encoding the intermediate enzymes Cyp8b1 (-43 ± 6%, nominal $p=0.0028$), Amacr (-31 ± 3%, nominal $p=0.043$) and Acox2 (-44 ± 9%, nominal $p=0.0228$), however no change in expression was observed for Cyp7a1, the rate-limiting enzyme in bile acid production. The metabolic links between these four pathways is highlighted in Fig. 4.3d. The data suggests a downregulation of the metabolic pathways leading to cholesterol, as well as downregulation of the cholesterol utilisation pathways leading to both steroid hormone and bile acid production. This finding may in part explain the regulation of glucose production observed following PAMN-treatment. The production of bile acid in the liver, and downstream signalling through the
farnesoid x receptor (Fxr) has been shown to regulate the expression of genes involved in glucose production including glucose-6-phosphatase (G6Pase) [241] and Pepck [242, 243], thereby lowering hepatic glucose production.

4.3.2.3. **Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified regulation of multiple lipid metabolic pathways**

In the sphingolipid metabolism pathway, there was an increase in PAMN-treated cells compared with PA-treated cells in the expression of one of the genes that converts ceramide to sphingosine (alkaline ceramidase 2, Acer2, +36 ± 10%, nominal $p=0.0086$), which can then be further converted to sphingosine-1-P. The enzyme that dephosphorylates sphingosine-1-P to sphingosine (and also dephosphorylates ceramide-P to ceramide) was decreased with PAMN-treatment (phosphatidic acid phosphatase type 2B, Ppap2b, -36 ± 6%, nominal $p=0.0014$). These transcriptional changes indicate a possible shift towards sphingosine-1-P metabolism away from ceramide production. The sphingosine-1-phosphate receptor SIP2 has been shown to activate Erk1/2 [244] regulated phosphorylation and stabilisation of the small heterodimer partner (SHP) [245], a transcriptional repressor that suppresses hepatic gluconeogenetic gene expression [246].

The linoleic acid metabolism pathway and arachidonic acid pathways identified increased expression of phospholipase group 2 1b (Pla2g1b) – which was significantly decreased with PA-treatment (-31 ± 4%, nominal $p=0.0120$) and increased with PAMN-treatment (+135 ± 13%, nominal $p=1.0x10^{-9}$). In contrast to
my findings, \( \text{Pla}2\text{g}1b^{-/-} \) mice show resistance to diet induced obesity [230] and reduced postprandial hyperglycaemia [228]. The \( \text{Pla}2\text{g}1b \) decrease observed with PA-treatment may therefore act as a cellular defence mechanism to reduce fatty-acid uptake and avoid lipid toxicity. Following PAMN-treatment, it is possible that this defence is no longer required, and therefore \( \text{Pla}2\text{g}1b \) expression increased. The cytochrome P450 2e1 was reduced following PAMN-treatment (\( \text{Cyp}2\text{e}1, -36 \pm 9\% \), nominal \( p=0.0480 \)). This enzyme has been found to display increased activity in the liver of type 2 diabetic patients [247], and \( \text{Cyp}2\text{e}1^{-/-} \) mice have been shown to be resistant to diet-induced obesity and insulin resistance [248], consistent with the decrease in the mRNA levels of this enzyme in PAMN-treated cells.

The regulation of the retinol metabolic pathway also shows direct links to glucose production, as all-\( \text{trans} \)-retinoic acid (a vitamin A derivative) has been shown to activate transcription of \( \text{Pepck} \) through activating transcription factor-2 (Atf2) [249]. The key regulator of retinoic-acid, \( \text{Cyp}2\text{6a}1 \), was increased with PAMN-treatment (+72 \pm 15\%, nominal \( p=0.0003 \)) and therefore may act to reduce availability of retinoic acid with PAMN treatment and lower \( \text{Pepck} \) expression and subsequently reduce glucose production. However, when \( \text{Pepck} \) (and \( \text{G6Pase} \)) mRNA expression levels were measured via RT-PCR (see Chapter 5), there was no difference between PA- and PAMN-treated cells.
4.3.2.4. **Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified transcriptional regulation of PPAR signalling**

Gene expression levels of the PPAR signalling pathway was regulated with the addition of metformin and sodium salicylate to the PA-treated cells (Fig. 4.4). The intestinal fatty acid binding protein *Fabp2* and brain fatty acid transport protein *Fabp7* were decreased (-25 ± 5%, nominal *p*=0.0220; -63 ± 3%, nominal *p*<0.0001) with PAMN-treatment compared with PA-alone-treated cells, while the main adipocyte fatty acid binding protein *Fabp4* was increased (+70 ± 11%, nominal *p*=0.0003). The main liver fatty acid binding protein in the liver however is *Fabp1*, which was not altered with either treatment, so the impact of the changes of the additional lower expressed proteins on fatty acid transport in the liver is not clear.

The mRNA expression level of *PPARδ* itself was decreased (-24 ± 5%, nominal *p*=0.0240) following PAMN-treatment.

The downstream transcriptional targets of all three PPARs were altered (see data in Fig. 4.4), with down-regulation of genes with diverse roles in ketogenesis (*Hmgcrs2*), lipid transport (*Apoa5*), lipogenesis (*Fads2*), cholesterol metabolism (*Cyp8b1*), and gluconeogenesis (*Pepck*, -23 ± 6%, nominal *p*=0.0193), which correlates with the lowering of glucose production following PAMN-treatment. Genes with roles in fatty acid oxidation were both up- (*Ehhadh, Acaa1*) and down-regulated (*Cyp4a2*). Signalling through this pathway could play a role in inducing the changes observed in the fatty acid metabolic pathways discussed above, as well as explaining the observed reduction in glucose production with PAMN-treated cells due to the decreased *Pepck* expression observed in the microarray data.
FIGURE 4.4: Transcriptional regulation of the PPAR signalling pathway between PA-and PAMN-treated cells.

Relative amounts of each transcript reported as percentage change from PA to PAMN, represented as mean ± SEM. All expression changes detailed were significant at nominal $p<0.05$. 

- VLDL chylomicron
- Fatty acid ligands
- Synthetic ligands

![Diagram showing regulation of PPAR signalling pathway]

- **Ligands**
  - **Transcription factors**
  - **Target genes**

- **Ppara** (no change)
- **Ppar6** -24 ± 5% ($p=0.0240$)
- **Ppara** (no change)

- **Fabp2** -25 ± 5% ($p=0.0220$)
- **Fabp4**(Ap2) +70 ± 11% ($p=0.0003$)
- **Fabp7** -63 ± 3% ($p=8.0x10^{-7}$)

- **Hmgcs2** -27 ± 6% ($p=0.019$)
- **Apoa2** +51 ± 17% ($p=0.0200$)
- **Apoa5** -48 ± 3% ($p=5.1x10^{-4}$)
- **Fads2** -25 ± 6% ($p=0.0239$)
- **Cyp8b1** -43 ± 6% ($p=0.0028$)
- **Ehdah** +108 ± 24% ($p=0.0004$)
- **Cyp4a2 (Cyp4a11)** -28 ± 8% ($p=0.0253$)
- **Aca1** +15 ± 5% ($p=0.0356$)
- **Acox2** -44 ± 5% ($p=0.0226$)
- **Angpt4** +36 ± 5% ($p=0.0043$)
- **Fabp4**(Ap2) +70 ± 11% ($p=0.0003$)
- **Sorbs1** +17 ± 4% ($p=0.0046$)
- **Pepck** -23 ± 6% ($p=0.0193$)
4.3.2.5. Gene set enrichment analysis between PA- and PAMN-treated FAO cells identified transcriptional regulation of multiple pathways involving immune function

When comparing vehicle versus PA-treated cells, GSEA identified regulation of the toll-like receptor signalling pathway, leukocyte transendothelial migration, antigen processing and presentation and the NOD-like receptor signalling pathways. These pathways regulate the production of pro-inflammatory cytokines and activate lymphocyte chemotactic effects. In contrast, the comparison of PA- versus PAMN-treated expression levels identified regulation of RIG-1 like receptor signalling pathway, systemic lupus erythematosus and the compliment and coagulation cascade pathways.

RIG-1-like receptor signalling pathways lead to the production of inflammatory cytokines following viral RNA detection by the cell. Similarly to the reduction in the complement factors in the complement and coagulation cascade pathway (see Appendix 5), there was a decrease in multiple genes involved in the innate immune response including DEAD box polypeptide 58 (Ddx58, -22 ± 6%, nominal p=0.0159) and interferon regulatory factor 7 (Irf7, -25 ± 5%, nominal p=0.0061), which normally activate the downstream induction of cytokine expression of interleukin 6 (IL-6) [250] and interferon-β (IFN-β) [251]. Due to the decreased levels of these pro-inflammatory genes, there may be potential for reduced type 1 interferon and inflammatory cytokine production with PAMN-treatment in this model – consistent with the main role of sodium salicylate in reducing cellular inflammation.
4.3.2.6. Gene set enrichment analysis between PA-and PAMN-treated FAO cells identified transcriptional regulation of mediators of oxidative stress

Glutathione metabolism tightly controls one of the key oxidative stress response molecules - glutathione. Glutathione interacts with glutathione peroxidase (Gpx1) to detoxify hydrogen peroxide [252]. Gpx1 expression was decreased (-37 ± 4%, nominal p<0.0001) with PAMN-treatment, indicating a reduced capacity to counter radical species, suggesting a possible reduction in oxidative stress with PAMN-treatment.

Nicotinate and nicotinamide metabolism regulates the production and recycling of NADP+, which itself is a by-product of glutathione cycling back to its antioxidant form [253]. The rate limiting enzyme for NAD⁺ biosynthesis, nicotinamide phosphoribosyltransferase (Nampt), was significantly increased with metformin and sodium salicylate treatment by +20 ± 7% (nominal p=0.0312). The increased availability of NAD⁺ via Nampt upregulation has been proposed as an alternative mechanism of action for the inhibitory effect of metformin on glucose production ([194], reviewed in [254]).

Related to these pathways is porphoryn and chlorophyll metabolism, which leads to the production of heme and bilirubin – a component of bile acid and potent antioxidant. The expression levels of four genes identified in this pathway, which lead to bilirubin production, were all decreased – Alas1 (-28 ± 3%, nominal p=1.6x10⁻⁵), Alad (-13 ± 4%, nominal p=0.0250), Uros (-16 ± 4%, nominal p=0.0437) and Blvra (-17 ± 5%, nominal p=0.0372). Further to a potential decreased capacity
for bilirubin production, the three glucuronosyltransferase genes which metabolise bilirubin into bilirubin β-diglucuronide (the water soluble form) were increased – *Ugt2b1* (-25 ± 7%, nominal $p=0.0193$), *Ugt2b10* (-29 ± 8%, nominal $p=0.0317$) and *Ugt2b4* (-25 ± 8%, nominal $p=0.0463$). Therefore, the availability of bilirubin may be decreased following PAMN-treatment in this model.

These three pathways taken together appear to indicate a selective shift away from an oxidative stress- response in this model following metformin and sodium salicylate treatment. The role of sodium salicylate in reducing fatty acid-induced inflammation [91] and oxidative stress [255] in the liver has been established, and one possible explanation may be that treatment with sodium salicylate reduces the need for such antioxidant responses by reducing the source of inflammation.
4.4. Discussion
The most convincing evidence for impairment of a metabolic pathway was found for acetyl-CoA to cholesterol metabolism. The identification of the terpenoid backbone, steroid, steroid hormone and primary bile acid biosynthesis pathways as being regulated following PAMN-treatment correlates with the known role of metabolites of these pathways in insulin action and hepatic glucose production. There was a general decrease in the expression levels of all members of the metabolic pathways regulating the synthesis of cholesterol from acetyl-CoA with PAMN-treatment. In addition, the transcriptional changes associated with multiple lipid and amino acid metabolic pathways with PAMN-treatment indicates a potential for increased generation of acetyl-CoA. Further, the metabolic pathway regulating the metabolism of primary bile acids from cholesterol was identified as being regulated following PAMN-treatment.

The role of acetyl-CoA in increasing hepatic glucose production via activation of pyruvate carboxylase has been detailed in Chapter 1. This mechanism may in part explain the increase in glucose production observed following PA-treatment in the current study. It is therefore curious that the changes in gene expression for the regulatory pathways leading to the generation of free acetyl-CoA suggest an increased ability for synthesis following PAMN-treatment. If this were the case, then there exists at least the following explanations as to why glucose production falls in PAMN-treated cells with the potential for increased acetyl-CoA production: (i) the generation of acetyl-CoA has already reached its maximum, limited by substrate availability, or (ii) the levels of acetyl-CoA do increase with PAMN-treatment, however the effects of PAMN-treatment are sufficient to overcome the acetyl-CoA
induced increase in pyruvate carboxylase activity in order to lower hepatic glucose production. In order to fully investigate this hypothesis, the levels of intracellular acetyl-CoA would have to be determined.

The comprehensive decrease in gene expression in the metabolic pathways regulating the conversion of cholesterol from acetyl-CoA suggests a decreased ability to synthesise cholesterol in PAMN-treated cells. Cholesterol has been shown to play a key role in the regulated uptake of long chain fatty acids across multiple cell types [256, 257]. The long chain fatty acid transport protein cluster of differentiation 36 (CD36/FAT) resides within the plasma membrane, and its association with cholesterol rich lipid rafts are required for activation of fatty acid transport [258]. This process has been shown to be cholesterol dependant in human HepG2 hepatoma cells, in which the cholesterol binding agent filipin III (which preferentially removes cholesterol from the plasma membrane, thus depleting caveolae lipid rafts [259]) reduced the uptake of $^3$H-oleic acid following 30 mins of pre-treatment [256]. This regulation by cholesterol of fatty acid transport may play a role in the decreased glucose production observed in PAMN-treated cells in the current study. By inhibiting the uptake of PA, the acetyl-CoA mediated increase in glucose production could be prevented. Further investigation into the regulation of cholesterol metabolism is therefore required to determine whether this mechanism may be playing a role in the regulation of glucose production in PAMN-treated cells. This will be further analysed in Chapter 5.
The role of bile acids and cholesterol metabolism in the regulation of glucose and lipid metabolism has been widely reported in recent years. A recent study by Meissner et al found that sequestration of bile acids in \( db/db \) mice using Colesevelam HCl for two weeks led to increased glucose clearance in the peripheral tissues by \( \approx 37\% \), but there was no effect on total hepatic glucose production [260]. Alternatively, in Zucker \( ^{fa/fa} \) rats it has been reported that activation of the nuclear bile acid Farnesoid X Receptor (Fxr), via the Fxr ligand 6\( \alpha \)-ethyl-chenodeoxycholic acid, leads to a reduction in blood glucose levels through reduced hepatic \( \text{Pepck} \) and \( \text{G6Pase} \) gene expression levels [243]. Likewise, synthetic agonists of Fxr, as well as hepatic overexpression of Fxr, have also been shown to reduce blood glucose levels, although this was reported with an increase in \( \text{PEPCK} \) expression alongside decreased \( \text{G6Pase} \) expression [242]. In vivo transgenic overexpression of cholesterol 7\( \alpha \)-hydroxylase in the liver – the rate limiting step in cholesterol to bile acid synthesis – has been shown to lead to an increased bile acid pool and subsequent decrease in blood glucose levels, correlating with decreased hepatic \( \text{G6Pase} \) expression, and no change in \( \text{PEPCK} \) expression [241]. The regulation of glucose homeostasis by bile acids therefore appears to be tissue specific, with sequestration away from peripheral tissues leading to improved glucose clearance, however direct effects of bile acids on the liver through its effects mediated by Fxr signalling lower glucose production. Investigation into the metabolism and synthesis of cholesterol and bile acids by PA- and PAMN-treatment in FAO cells is required to determine whether they are contributing to glucose homeostasis in this model. This will be further analysed in Chapter 5.
There have been relatively few studies looking into the genome wide effects of PA in hepatic cell or tissue models. Swagell et al. [235] found differential regulation of 162 of 18,000 genes using cDNA microarrays in the human hepatic Huh-7 cells following 48 h treatment with 0.15 mM PA. The total number of regulated genes was far fewer than identified in the current study, most likely due to a smaller number of replicate samples; n=4 in both control and PA treated cells. While the Swagell study did not perform GSEA, differentially expressed genes were grouped according to function and described. Amongst their key findings were increased expression of mediators of lipid and cholesterol transport, fatty acid metabolism and oxidative stress. Given that multiple pathways with similar functions in fatty acid and cholesterol metabolism were down-regulated in the current study following PAMN-treatment, these pathways appear to be correlated with PA-induced increases to glucose production. Additionally, expression of many mediators of cellular proliferation were found in both the Swagell [235] and this study to be decreased in response to PA-treatment. Swagell [235] observed a correlation between these changes in gene expression and decreased cellular number following PA treatment for 48 h. Further complicating the correlation between reduced proliferation and glucose production however is the finding that reduced glucose production by metformin is also associated with decreased protein synthesis and cellular proliferation through AMPK activation ([261], reviewed in [262]). It is therefore unclear whether the reduction in cellular proliferation plays a role in the increased production of glucose by PA treatment as observed in this model.
Using human HepG2 hepatoma cells treated with 50 μM PA for 24 h, Vock et al. [234] performed cDNA microarray analysis and found only 11 of 38,500 array probes to be regulated by PA-treatment. This is strikingly lower than the number of regulated genes in the current study, however given the reduced PA concentration, time of exposure and number of samples (n= 3 independent experiments, each in triplicate), a reduced number of regulated genes with increased sensitivity to PA-treatment might be expected. The main regulated genes identified were increased expression of the inhibitor of fibrinolysis Serine peptidase inhibitor E1 (Serpine1) and increased expression of insulin-like growth factor II (Igf-II), which has been linked to the induction of type 2 diabetes in mouse models of Igf-II overexpression [263].

In contrast to these in vitro based microarray studies, this study has identified pathway regulation of inositol phosphate signalling with PA-treatment, with reduced Impk expression potentially reducing insulin signalling through Akt and thus supressing the effects of insulin on repression of glucose production [240]. The effects of regulation of this pathway on phosphorylation of PIP₂ – PIP₃ generation, coupled with the widespread changes to many lipid metabolic genes with PAMN treatment warrants further investigation.

Regulation of signalling pathways controlling cellular inflammation were induced by both PA- and PAMN-treatments, and included regulation of the JNK signalling pathway and increased expression of Fos and Jun – mediators of inflammatory cytokine release and recruitment of lymphocytes. Given the role of macrophage infiltration and subsequent inflammatory cytokine release compounding pre-
existing insulin resistance in peripheral tissues [63, 264, 265], the control of inflammatory pathways is important in the restoration of altered glucose metabolism. A reduction in the expression of key upstream mediators of NF-κB signalling (\textit{Ddx58} and \textit{Irf7}) was observed. Alongside this reduction in gene expression is the established inhibition of Ikkβ by sodium salicylate, which has been shown in animal models of diet-induced insulin resistance to improve abnormal liver Irs-1 (increase tyrosine phosphorylation, reduce Ser 307 phosphorylation), Irs-2 (increase tyrosine phosphorylation, reduce Ser 233 phosphorylation) and Akt (increase Ser 473) phosphorylation [91] and lower hepatic glucose production, fasting blood glucose and fasting insulin levels [255]. It is therefore likely that the regulation of inflammatory signalling pathways is playing a role in the effects of PA- and PAMN-treatment on glucose production in this model.

The regulation of oxidative stress pathways observed in response to PAMN treatment has been previously linked to the hepatic glucose production lowering mechanisms of both metformin and sodium salicylate. Sodium salicylate has been shown to reverse lipid-induced increases in release of plasma malondialdehyde (MDH) a marker of whole body oxidative stress, as well as increased liver glutathione peroxidase (Gpx) activity in a rat model of intralipid induced whole body insulin resistance [255]. In contrast to these findings, this study observed a decrease in Gpx1 gene expression levels in response to PAMN treatment. Whether these differences are due to PAMN-treatment reducing the stimulus for Gpx1 transcription in the \textit{in vitro} model versus the more complex \textit{in vivo} model is unclear. Metformin has also been shown to induce transcription of oxidative stress response genes, specifically through AMPK-induced transcription of \textit{Nampt}, \textit{Gcn5} and \textit{Sirt1}
The proposed mechanism involves a metformin-induced increase in NAD$^+$-dependant Sirt1 inhibition of Torc2-mediated glucose production, while simultaneously increasing Gcn5 inhibition of Pgc1α-stimulated glucose production. In the current study, although no changes to Sirt1 or Gcn5 expression was observed, Nampt expression was increased by 20 ± 7% with PAMN-treatment ($p=0.0312$ compared with PA-treated) which remains consistent with the published study.

The regulation of the PPAR signalling pathway, as identified in the GSEA analysis, may explain the regulation of the numerous fatty acid signalling pathways observed in our model. Activation of PPARs α and γ have been attributed to both saturated and unsaturated fatty acids [266]. Activation of the PPARα with the glucocorticoid dexamethasone or the agonist WY14,643 has been shown to lead to dysregulation of glucose production in the liver, increasing both Pepck and PPARα expression in primary human hepatocytes [267]. Further downstream targets of PPARα identified in the current study include Fads2, Acox2, and Fabp4. The identification of transcriptional regulation of the 9 pathways via GSEA involving fatty acid and acetyl-CoA metabolism may be explained, in part, by this interaction.

Despite the wealth of biologically relevant information that can be obtained via microarray and GSEA approaches, the main drawback to this technology is the distal nature of gene expression to metabolic outputs. Multiple factors including regulation of translational processes, post-translational modifications, protein turnover, availability of co-factors and signalling initiation all confound the interpretation of gene expression data. Therefore, there is a need for further analysis in order to confirm any pathway regulatory changes, which can be
confirmed by measuring relevant key metabolites. This will be further investigated in Chapter 5.

In summary, a GSEA approach was used to investigate the regulation of metabolic and signalling pathways in both the PA-induced dysregulation of glucose production, as well as its restoration with PAMN-treatment. The findings of this chapter, including regulation of multiple lipid metabolic pathways including phosphatidylinositol and cholesterol metabolism, however have not yet been confirmed beyond the level of gene expression changes. Therefore, Chapter 5 will investigate the changes to the global lipid profile in vehicle-, PA- and PAMN- treated FAO cells in order to further investigate this question.
Chapter 5 – PA-induced alterations to the global lipidomic profile in FAO hepatoma cells

5.1. Introduction

In Chapter 4, the reversal of PA-induced high glucose production in the liver by PAMN-treatment was characterised by transcriptional regulation of a number of metabolic pathways regulating fatty acid metabolism. In addition, a comprehensive decrease in the gene expression levels of all of the metabolic enzymes regulating the synthesis of cholesterol from acetyl-CoA was observed. Given the accepted role of many lipid metabolites in the pathogenesis of T2D – most predominately DAGs [268] and ceramides [152] - further analysis at the level of the individual lipid metabolites was warranted to observe the effect of these transcriptional changes at the level of lipid metabolism. This approach may identify potential mediators of dysregulated glucose production in this model. Global lipidomic approaches, made possible with current generation chromatographic and mass spectroscopic technology, provide the means to analyse these changes and track the metabolism of specific intermediates of interest [122]. These new approaches to metabolomics investigation are proving as revolutionary to the field as microarray technology has been in the study of transcriptional regulation. By allowing the study of nearly the entire cellular lipid content, improved analysis on the regulation of multiple pathways can be performed. Given the complex nature of T2D, the ability to separate out multiple dysregulated metabolic pathways will prove critical in furthering understanding of the disease.
Global lipidomic approaches to dissecting the mechanisms of T2D are becoming more widespread as the identity of specific lipid species in the regulation of glucose metabolism are being recognised rather than whole classes. For example, the activation of total PKC in HepG2 hepatoma cells following acute (1 – 12 h) fatty acid treatment was found to be proportional to the degree of unsaturation of the fatty acid [269]. Activation of total PKC in lipid vesicles in vitro by DAG species has been shown to be greater for DAGs containing PUFA side chains such as 18:0/22:6 and 18:0/20:4 than the saturated DAG 18:0/18:0 [270]. Further, DAG activation of conventional (α, βl and βll) and novel (δ, ε, η and θ) PKC isoforms is structure-specific, requiring sn-1,2-DAG species, and is unaffected by sn-1,3-DAG and sn-2,3-DAG [271]. In muscle cells, the ceramide class of lipids have been shown to play a key role in the induction of insulin resistance [152], however levels of total ceramides have been shown to remain relatively constant between healthy insulin sensitive patients and obese insulin resistant patients [272]. One study has even described a positive correlation between skeletal muscle total ceramide content and insulin sensitivity in individuals [272]. It is therefore likely that this discrepancy can be explained by the role of individual lipids on insulin action and glucose metabolism, rather than the class as a whole. Determining the individual lipid species within those classes that are responsible for these toxic effects is the next challenge in determining new targets for therapeutic intervention.

Much of the published work on utilising lipidomic approaches has been on profiling the plasma lipids of obese cohorts and comparing with lean healthy control subjects to identify novel markers of insulin resistance and obesity ([273], reviewed in [122]). However, since not all obese individuals are insulin resistant, or develop T2D,
careful analysis of such markers is required to determine whether an association with insulin resistance can be made. There have been studies attempting to answer this problem, for example identifying TAG species in varying lipoprotein fractions to identify species which correlate with insulin resistance [274]. Common criticisms of such studies have been a failure to perform adjustments for obesity and as such it is not clear if some of the associations made with insulin resistance or obesity are truly independent of one another. Associations with a number of plasma markers including saturated TAG species [274] and ceramides [273] have been described, however similar studies on patient intra-hepatic lipid metabolism are less common.

The key finding of Chapter 4 was that the gene expression levels of every enzyme involved in the synthesis of cholesterol from acetyl-CoA were decreased in PAMN-treated cells compared with PA-treated cells. In addition, numerous lipid metabolism pathways were identified as being differentially regulated in PAMN-compared with PA-treated cells. Finally, the regulation of phosphatidylinositol (PI) metabolism as being differentially expressed between vehicle- and PA-treated cells was a novel finding. Therefore, the primary aim of this chapter was to complete a mass-spectroscopic based lipidomic analysis of vehicle, PA- and PAMN-treated cells in order to determine if the changes observed in transcriptional regulation as outlined in Chapter 4 are related to an altered lipid profile in these cells (a “whole lipidome” approach). The secondary aim of this chapter was to investigate specific aspects of lipid metabolism (a “targeted lipidome” approach) highlighted in the findings of Chapter 4 including cholesterol and bile acid metabolism in order to further characterise the mechanisms involved in the dysregulation of glucose metabolism.
5.2. Methods

5.2.1. Lipidomic profiling of FAO cells following PA- and PAMN-treatment

Cell lysate for lipidomic profiling was collected from vehicle- (n=6), PA- (n=6) or PAMN- (n=6) treated FAO hepatoma cells, in 10 cm dishes with a media volume of 10 mL. Treated cells (as detailed in Chapter 2) were trypsinised, and centrifuged at 200 x g for 5 min at room temperature then resuspended in RPMI 1640 media (Invitrogen), then centrifuged at 200 x g for 5 min at room temperature, washed twice in 500 μL 1 x PBS pH 7.4 and transferred to a 1.5 mL tube pre-chilled to 4°C. On ice, cells were lysed using a sonicator probe at 20% amplitude (model 250 Branson Sonifier®, with model 102c sonicator probe) twice for 15 seconds, put back on ice for 1 min following each sonication. Samples were centrifuged at 14,000 x g for 5 min at 4°C, and supernatant (cell lysate) was collected and stored at -80°C. Protein concentrations for each sample were determined using the Pierce BCA Protein Assay Kit, and each sample was diluted in 1 x PBS pH 7.4 to achieve equivalent protein concentration for all samples (2.0 mg/mL).

The following lipid extraction technique and mass-spectroscopic analysis were performed at Professor Peter Meikle’s Metabolomics laboratory (BakerIDI, Melbourne, AUS) following established protocols developed for comprehensive lipid analysis in human plasma samples [275]. Lipid extraction from cell lysates were performed using a modified Folch extraction [276]. 10 μL of cell lysate was used,
and an internal standard (ISTD) mix (15 μL, consisting of 28-lipid species standards mix) were added into the samples. 2 mL of 2:1 chloroform:methanol solution was added and samples were placed on a rotary mixer for 10 min at room temperature followed by sonication for 30 min and incubation at room temperature for 20 min. Samples were centrifuged at 16,000 x g for 10 min at room temperature and the supernatant dried down under nitrogen at 40 °C. Samples were resuspended in 50 μL saturated butanol and sonicated for 10 min. 50μl MeOH containing 10 mM ammonium formate was then added and centrifuged again at 2000 x g for 5 min at room temperature. The supernatant was transferred to 0.2 mL micro-inserts in 32x11.6 mm glass vials with Teflon insert caps.

Liquid chromatography, electrospray ionisation-tandem mass spectrometry (LC ESI-MS/MS) analysis was performed by Ms Jacqui Weir at Professor Peter Meikle’s Metabolomics laboratory (BakerIDI, Melbourne, AUS). Separation was achieved using a HP1200 liquid chromatography system with a Zorbax C18 column (1.8 μm, 50 x 2.1 mm column, Agilent technologies, USA). The mobile phase was tetrahydrofuran:methanol:water in a 30:20:50 ratio (A) and 75:20:5 (B) both containing 10 mM NH₄COOH. The following gradients were used for all lipids except for DAGs and TAGs; 100% A/0% B reducing to 0%A/100%B over eight min followed by 2 min at 0% A/100% B, a return to 100% A/0% B over 0.5 min then held for 3.5 min at 100% A/0% B prior to the next injection. DAGs and TAGs were separated using the same system with an isocratic flow at 15%A/85% for 6 min between injections. Electrospray ionisation-tandem mass spectrometry was then performed using an Applied Biosystems 4000 Q/TRAP mass spectrometer with turbo-ionisation source (350°C) using Analyst 1.5 software. Quantification of individual lipid species
was performed using scheduled multiple-reaction monitoring (MRM) in positive ion mode. Lipid concentrations were calculated by measuring the peak area of each lipid species (using Multiquant 1.2 software) and relating it to the peak area of the corresponding internal standard. Total lipids of each class were determined by summing the individual lipid species. Data is presented as an average of all sample measurements ± SEM.

A series of product ion scans in negative ion mode were performed to determine the side chains of PI 38:5 (see Appendix 5). Based upon previous characterisation work undertaken in Professor Peter Meikle’s Metabolomics laboratory at the Baker IDI, PI 38:5 has a known retention time of approximately 5.4 min for their mass spectroscopic platform. Following fragmentation of the sample at this specific retention time, each fragment of the lipid loses one hydrogen atom and becomes negatively charged. The charged fragments were then compared with standard fragment sizes for the identification of the side chains of interest (resolution is measured with an error of ± 0.4 mass units).

### 5.2.2. Analysis of excreted cholesterol levels in conditioned media

Conditioned media was collected from vehicle- (n=3), PA- (n=3) or PAMN- (n=3) treated FAO hepatoma cells in 10 cm dishes with a media volume of 10 mL. Total cholesterol were measured via HDL and LDL/VLDL Cholesterol Assay Kit (Abcam, Cambridge, UK), following the manufacturer’s instructions. Briefly, in a 1.5 mL tube, 100 μL conditioned media was mixed with 100 μL precipitation buffer (kit supplied reagent), incubated for 10 min at room temperature, then centrifuged at 2000 x g
for 10 min at room temperature. The supernatant (HDL fraction) was transferred to a fresh 1.5 mL microfuge tube, while the precipitate (LDL/VLDL) was resuspended in 200 μL of 1 x PBS pH 7.4. 20 μL of the HDL, LDL/VLDL fractions, and unfractionated conditioned media (for total cholesterol measurement) was added to 80 μL of reaction buffer (kit supplied reagent, containing cholesterol oxidase and a H₂O₂ sensitive probe) in the presence or absence of cholesterol esterase to measure total (with cholesterol esterase) and free (without cholesterol esterase) cholesterol. The reaction was incubated for 60 min at 37°C and protected from light. Absorbance was read at 570 nm and compared with a series of known standards prepared in cholesterol assay buffer using 0.25 μg/μL cholesterol standard supplied with the assay kit. The subtraction of free from total cholesterol levels yields measurement of cholesterol esters.

5.2.3. Measurement of excreted and intracellular total bile acids

Cell lysate for total bile acid analysis were extracted from FAO cells treated with vehicle (n=3), PA (n=3) or PAMN (n=3) as described above for lipidomic cell lysate preparation. Conditioned media was also collected to measure extracellular total bile acids as described above for analysis of excreted cholesterol levels. Total bile acids were measured via Total Bile Acid Assay kit (enzyme cycling method, DZ042A-K, Diazyme Labs, USA), following the manufacturer’s instructions. Briefly, 8 μL of sample conditioned media or cell lysate was incubated with 270 μL assay reagent 1 (kit supplied reagent) for 5 min at 37°C, followed by the addition of 90 μL of assay reagent 2 (kit supplied reagent). Absorbance was then immediately measured at 405 nm for two min at 37°C. The rate of change for the reaction was measured
from 60 – 120 seconds. The rate of change is directly proportional to the level of total bile acids present, and was compared with a series of known standards prepared in 1 x PBS pH 7.4 using 50 μM total bile acid standard supplied with the assay kit.

5.2.4. RNA extractions and RT-PCR analysis of gluconeogenetic genes
Total RNA was extracted and quantified from vehicle- (n=4), PA- (n=4) or PAMN- (n=4) treated FAO hepatoma cells as described in Chapter 3 (technical replicates), in addition to vehicle- (n=4), PA- (n=4) or PAMN- (n=4) treated FAO hepatoma cells (biological replicates). RT-PCR was performed as described in Chapter 3. Data is presented as the average of n=8 samples. Primer sequences are detailed in Table 5.1 below. All primers were designed using Beacon Designer 7.2 (Premier Biosoft International, CA, USA).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pepck</td>
<td>AGCCATGTGCAACTCATGCA</td>
<td>CTCGGTGCCACCTGAAACA</td>
</tr>
<tr>
<td>G6Pase</td>
<td>ACGCCTTCTATGTCTCTTTC</td>
<td>TGTTGCTGTAGTAGTCGGTGCTCC</td>
</tr>
</tbody>
</table>

*Pepck*, phosphoenolpyruvate carboxykinase, NM_198780; *G6Pase*, glucose-6-phosphatase, NM_013098.
5.2.5. Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM). Data were first analysed for normality using a one-sample Kolmogorov-Smirnov test. All data was determined to be normally distributed. Data were then either analysed using independent samples Students T-test, or a one-way ANOVA. Homogeneity of variance was determined using Levene’s Test, and post-hoc analysis of ANOVA used either Fisher’s least significant difference (LSD, for homogeneous variance) or Games-Howell (for non-homogeneous variance). Data were considered significant at $p<0.05$. All data reported as mean values ± SEM.
5.3. Results

As outlined in Chapter 4, GSEA of regulated expression between PA- and PAMN-treated cells highlighted widespread transcriptional regulation of pathways involved in lipid metabolism. The most prominent of these regulatory pathways linked acetyl-CoA metabolism through to cholesterol metabolism, in which the majority of genes were down-regulated (from terpenoid backbone biosynthesis, hormone biosynthesis and steroid hormone biosynthetic pathways). Linked to this was the observed down-regulation in the primary bile acid biosynthetic pathway. Mass spectroscopic analysis of common lipid species was therefore performed to assess whether the observed changes at the transcriptional level may be having an effect on lipid metabolism, and therefore affecting specific lipid species.

The following mass-spectroscopy derived results have been provided in pmol/mg protein for each lipid class. In order to directly compare between lipid classes, a complete set of standards is required for each individual lipid species measured. It should be noted that there were only 1-3 internal standards used for each lipid class in this study. Therefore, as advised by Prof Peter Meikle of the Metabolomics Laboratory at the Baker IDI, the comparisons of relative lipid levels have been restricted to within a single class of lipid.
5.3.1. Regulation of the TAG lipid profile by palmitate, metformin and sodium salicylate

Following PA treatment, the levels of total TAG species were increased by +175 ± 52% compared with vehicle-treated cells (nominal \( p=0.0360 \)), and remained unchanged following PAMN-treatment (compared with PA-treated cells, Fig. 5.1 a). Palmitate treatment led to increased levels of 21 different TAG species, all with side chains ranging from 14C-18C, and zero to two double bonds (Fig. 5.1 b). Only 9 of these lipids contained a PA side chain, indicating that PA-treatment is driving metabolism into other medium or long chain species that are stored as TAGs. Following PAMN-treatment, only two TAG species were reduced (Fig. 5.1 b) – 18:0 18:1 18:1 (-65 ± 22%, nominal \( p=0.0431 \)), and 18:0 18:0 18:1 (-75 ± 16%, nominal \( p=0.0288 \)) when compared with PA-treated cells. A further ten TAG species were detected which did not significantly change following either PA- or PAMN-treatment (Fig. 4.1 c).
FIGURE 5.1: Regulation of intracellular TAG species with PA- and PAMN-treatment in FAO hepatoma cells.

Intracellular TAG levels in vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) Increased total intracellular DAGs are observed with PA. (b) Individual TAG species with changed levels following treatment with either PA or PAMN. (c) Individual TAG species which did not change after treatment with either PA or PAMN. Data are expressed as mean ± SEM, n=3, *p<0.05 versus vehicle-treated lipid levels, †p<0.05 versus PA-treated lipid levels.
5.3.2. Regulation of the DAG lipid profile by palmitate, metformin and sodium salicylate

Intrahepatic DAG species have been previously linked to dysregulation of hepatic glucose production in obese patients [268], therefore making DAGs a metabolite of interest in this study. Total DAG levels increased following PA-treatment by +36 ± 11% (nominal \( p=0.0433 \)) compared with vehicle-treatment. Similar to TAG levels, the increase in DAG levels was not changed with PAMN-treatment when compared with PA-treated levels (Fig. 5.2 a).

Of all the DAG species detected, four had increased levels following PA-treatment (Fig. 5.2 b) – 16:1 18:1 (+64 ± 17%, nominal \( p=0.0350 \)), 18:1 18:2 (+57 ± 16%, nominal \( p=0.0315 \)), 18:1 18:1 (+50 ± 4%, nominal \( p=0.0019 \)), and 18:0 18:1 (+44 ± 13%, nominal \( p=0.0406 \)) compared with vehicle. Two of these DAG species, 18:1 18:1 and 18:0 18:1 were in turn decreased following PAMN-treatment (-18 ± 7%, nominal \( p=0.0320 \), and 36 ± 7%, nominal \( p=0.0304 \) respectively compared with PA-treated cells), making them of specific interest as the increased lipid levels were reversed in parallel with the reversal of dysregulated glucose production. One species – 18:1 20:0 – was unchanged with PA-treatment when compared with vehicle-treatment, but was decreased following PAMN-treatment (-49 ± 4%, nominal \( p=0.0216 \) compared with PA-treatment). The common inclusion of the side chain 18:1 (oleate) in all of these DAG species indicates a shift towards 18:1 DAG incorporation. Given the proposed role of DAGs in PKC activation and impairment of downstream insulin signalling, the increase in 18:1 containing DAGs may be a key regulator of glucose production in this model. A further 17 DAG species were measured which did not change following either PA or PAMN-treatment (Fig. 5.2 c).
Chapter 5: Lipidomic profiling

FIGURE 5.2: Regulation of intracellular DAG species with PA- and PAMN-treatment in FAO hepatoma cells.

Intracellular DAG levels in vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) Increased total intracellular DAGs were observed with palmitate treatment. (b) Individual DAG species with changed levels following treatment with PA or PAMN. (c) Individual DAG species which did not change following treatment with either PA or PAMN. Data are expressed as mean ± SEM, n=3, *p<0.05 versus vehicle-treated lipid levels, †p<0.05 versus PA-treated lipid levels.
5.3.3. Regulation of the cholesterol lipid profile by palmitate, metformin and sodium salicylate

Due to the transcriptional regulation of lipid metabolic pathways leading to cholesterol synthesis following PAMN-treatment (see Chapter 4), the levels of cholesterol and cholesterol esters were measured to determine whether regulation of the levels of these lipid species occurs in parallel with the regulation of hepatic glucose production. There was no significant difference in total intracellular cholesterol levels with either PA- (compared with vehicle-treated cells) or PAMN-treatment (compared with PA-treated cells; Fig. 5.3 a). PA-treatment for 48 h increased total cholesterol esters (+92 ± 26%, nominal $p=0.0092$ compared with vehicle-treated cells), and while there was a trend for reduction in the levels of cholesterol esters following PAMN-treatment this did not reach significance (-31 ± 16%, nominal $p=0.0830$, Fig. 5.3 b). As single species, only the levels of cholesterol ester 16:1 was significantly increased with PA-treatment (+180 ± 57, nominal $p=0.0127$, Fig. 5.3 c). There was a general trend for an increase across eight of the nine measured cholesterol esters (Fig. 5.3 c – d) – which was reflected in the observed increase to total cholesterol esters with PA-treatment. Only cholesterol ester 18:1 was altered with PAMN-treatment (-58 ± 13%, nominal $p=0.0225$, Fig. 5.3 c).

The lack of widespread changes to intracellular cholesterol species appears to be inconsistent with the regulatory changes observed in the transcriptional study in Chapter 4 in response to PAMN-treatment. However, because only total levels have been measured at this stage, alterations to metabolic flux through this pathway cannot be discounted.
FIGURE 5.3: Regulation of intracellular cholesterol and cholesterol ester levels in PA- and PAMN-treated FAO hepatoma cells.

Intracellular cholesterol levels vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) Total intracellular cholesterol levels were unchanged with either PA- or PAMN-treatment. (b) Total intracellular cholesterol esters were increased following PA-treatment. (c) Individual cholesterol ester species with changed levels following treatment with either PA or PAMN. (d) Individual cholesterol ester species that did not change after treatment with either PA or PAMN. Data are expressed as mean ± SEM, (a) n=3, (b-d) n=6, *p<0.05 versus vehicle-treated lipid levels, †p<0.05 versus PA-treated lipid levels.
5.3.4.1. Excreted cholesterol was detected at very low levels and was not regulated by either PA- or PAMN-treatment

As there was no observed changes to cholesterol metabolism in response to PAMN-treatment, levels of excreted cholesterol were measured to determine whether PAMN-treatment resulted in increased cholesterol release from the cells. Levels of HDL and LDL/VLDL cholesterol, as free cholesterol, esterified cholesterol or total cholesterol forms were measured. No significant differences were observed between any of the treatment groups (vehicle-, PA- or PAMN-treated; Fig. 5.4). In addition, despite a modest average %CV (19%), the data was at the low end of the assay’s detection limit, as the LDL/VLDL results cross the x-axis. The standards used for the assay ranged from 0.25 – 7 μg/well cholesterol, which yielded a standard curve with an $R^2=0.9983$. The highest concentration measured was 0.093 μg/well cholesterol in the vehicle total HDL group, approximately a third of the lowest standard. The reliability of the assay for the detection of excreted cholesterol in conditioned media was therefore difficult to interpret, however the standard deviation within each sample is modest and consistent. In light of the limitations reported here with regards to this assay, these results indicate no difference between excreted cholesterol levels in vehicle-, PA- or PAMN-treated cells.
FIGURE 5.4: Profile of excreted cholesterol following PA- and PAMN-treatment in FAO hepatoma cells.
Excreted HDL, LDL/VLDL and total cholesterol fractions in vehicle- (black), PA- (grey) and PAMN- (white) treated conditioned media. Data are expressed as mean ± SEM, n=3 *p<0.05 versus vehicle-treated cholesterol levels, †p<0.05 versus PA-treated cholesterol levels.
5.3.5. Total bile acids were not regulated by PA- or PAMN-treatment in FAO hepatoma cells

As there was no detectable difference in either intracellular or excreted cholesterol, bile acids were next measured to determine if changes in cholesterol pathway regulation might result in altered bile acid production. This arm of the cholesterol biosynthetic pathway was also identified via GSEA (see Chapter 4), and in addition the role of bile acids in regulating glucose metabolism through the Fxr receptor has been described previously [242, 243], therefore making the determination of both intracellular and excreted bile acid levels important in identifying potential mediators of glucose production in this model.

Both total intracellular and excreted bile acids were measured in cell lysate and conditioned media respectively. No differences were observed in the total intracellular or excreted levels of bile acids between vehicle-, PA- or PAMN-treated cells (Fig. 5.5). However the methodology used in this study for detection of total bile acids is only capable of measuring the total level of bile acid, and is unable to determine the rate of flux through cholesterol to bile acids. Therefore, the role of bile acids in regulating glucose production requires further investigation at this stage.
5.3.6. Modest regulation of the ceramide lipid profile by PA- and PAMN-treatment

Although ceramides have been shown to play a key role in the regulation of insulin resistance and glucose metabolism in muscle cells, their role in the liver is not as well defined (see Introduction). Therefore, characterisation of ceramide metabolism in this model was warranted. Total ceramide levels did not change with PA- or PAMN-treatment (Fig. 5.6 a). It is becoming increasingly clear however that specific species of ceramide have the ability to affect insulin signalling, not just total ceramide levels. There were four ceramide species that increased with PA-
treatment compared with vehicle-treated cells; cer 18:0 (+34 ± 8%, nominal 
p=0.0110), cer 20:0 (+123 ± 19%, nominal p=0.0001), cer 22:0 (+54 ± 11%, nominal 
p=0.0011), mono-hexocyl-ceramide (MHC) 20:0 (+242 ± 60%, nominal p=0.0175) 
and MHC 22:0 (+107 ± 23%, nominal p=0.0041), however these were not further 
altered following PAMN-treatment (Fig. 5.6 b). Only MHC 24:0 was decreased with 
PA-treatment (-19 ± 6%, nominal p=0.0363) compared with vehicle-treatment. 
Further, only one ceramide, dihydro-ceramide (dhcer) 20:0 was lowered with 
PAMN-treatment (-41 ± 8%, nominal p=0.0386) compared with PA-treated cells (Fig. 
5.6 b). A further 15 ceramide species remained unchanged following both PA- and 
PAMN-treatment (Fig. 5.6 c).
FIGURE 5.6: Regulation of intracellular ceramide species with PA- and PAMN-treatment in FAO hepatoma cells.

Intracellular ceramide levels in vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) Total intracellular ceramide remained unchanged with PA- and PAMN-treatment. (b) Individual ceramide species with changed levels following treatment with either PA or PAMN. (c) Individual ceramide species which did not change following treatment with PA or PAMN. Data are expressed as mean ± SEM, n=6, *p<0.05 versus vehicle-treated ceramide levels, †p<0.05 versus PA-treated ceramide levels.
5.3.7. Modest regulation of the sphingomyelin lipid profile by PA- and PAMN-treatment

Structurally related to ceramides, the sphingomyelins (SM) are a class of membrane phospholipids usually located within the membrane of the myelin sheath of nerve cell axons. Sphingomyelins contain a ceramide backbone and polar head group, and can be interconverted to ceramides. *Sphingomyelin synthase 2 (Sms2)* knockout animals have been shown to have lower fasting blood glucose and insulin levels alongside a reduced SM content in plasma membrane lipid microdomains [277, 278]. *In vitro* studies have shown the addition of SM inhibited insulin-stimulated Akt phosphorylation in HepG2 cells following acute (5 h) treatment [278]. In this study, total sphingomyelins were not altered between vehicle-, PA- or PAMN-treated cells (Fig. 5.7 a).

Following PA-treatment, SM 18:1 and 20:0 both increased (by +385 ± 78%, nominal $p=0.0386$ and +94 ± 24%, nominal $p=0.0278$ respectively) compared with vehicle-treated cells. In contrast to the effects of PAMN-treatment on the other lipid classes, two species of SM increased with PAMN-treatment compared with PA-treated cells; 14:1 (+201 ± 47%, nominal $p=0.0204$) and 20:1 (+150 ± 38%, nominal $p=0.0061$, Fig. 5.7 b). Although there was no overall change to SM lipids with either PA- or PAMN-treatment, the alterations of individual species may play a role in the regulation of hepatic insulin sensitivity with PA-treatment.
FIGURE 5.7: Regulation of intracellular sphingomyelin species with PA- and PAMN-treatment in FAO hepatoma cells. Intracellular sphingomyelin levels in vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) Total intracellular sphingomyelin remain unchanged with PA- and PAMN-treatment. (b) Individual sphingomyelin species with changed levels following treatment with either PA or PAMN. (c) Individual sphingomyelin species which did not change after treatment with either PA or PAMN. Data are expressed as mean ± SEM, n=6, *p<0.05 versus vehicle-treated lipid levels, †p<0.05 versus PA-treated lipid levels.
5.3.8. Regulation of the phosphatidylinositol lipid profile by palmitate, metformin and sodium salicylate

Given their role as signalling intermediates in the insulin signalling cascade, and the identification of the inositol phosphate metabolism pathway as being regulated with PA-treatment, the phosphatidylinositol (PI) class of lipids was investigated to observe if the changes in transcriptional regulation impacted upon the levels of intracellular metabolites of this class. Total PI levels were not significantly different following PA- or PAMN-treatment (Fig. 5.8 a). Phosphatidylinositol species were the only class of lipid where the majority of changes observed at the individual species level were due to PAMN- treatment compared with PA-treatment (Fig. 5.8 b). Treatment with PAMN lead to increased levels of PI 32:1 (+109 ± 38%, nominal \( p=0.0074 \)) and PI 36:4 (+55 ± 14%, nominal \( p=0.0296 \)), while also leading to decreased levels of PI 38:2 (-38 ± 9%, nominal \( p=0.0397 \)) and PI 40:5 (-24 ± 5%, nominal \( p=0.0414 \)), compared with PA-treated cells. PA-treatment increased levels of PI 36:1 (+49 ± 10%, nominal \( p=0.0013 \) compared with vehicle-treated cells), while levels of this species were decreased following PAMN-treatment (-35 ± 6%, nominal \( p=0.0010 \) compared with PA-treated cells).

One species of specific interest identified through this analysis was PI 38:5. PA-treatment lead to decreased levels of PI 38:5 (-34 ± 11%, nominal \( p=0.0283 \) compared with vehicle-treated cells). There was a trend for an increase in PI 38:5 following PAMN-treatment, although this did not reach significance (+36 ± 2%, nominal \( p=0.0691 \) compared with PA-treated cells). The regulation of PI phosphorylation by phosphoinositide-3-kinase (PI3K), and subsequent downstream activation of the insulin signalling cascade has been described for many years [279].
Despite this, the identity of specific PI species and their side chains and what role the identity of the side chains has in signal transduction or glucose production, has yet to be described. The potential to identify a specific PI species, which might play a key role in signal transduction involved in glucose production, would be an important advance in our understanding of this signalling molecule and insulin action. For this reason, further investigation as to the identity of the side chains of PI 38:5 was warranted, and this is detailed below.

Additionally, PI 40:4 decreased following PA-treatment (-32 ± 2%, nominal $p=0.0047$ compared with vehicle-treated cells), and remained unchanged following PAMN-treatment (mirroring the induction of insulin-resistance with PA-treatment, which was not restored with PAMN-treatment). However, reduction in levels of this lipid species only reached nominal significance upon repetition of the mass spectroscopic analysis (the addition of further 3 independent experiments bringing the total to 6), which occurred at the same time as the side chain determination of PI 38:5 was performed. Similarly the changes observed in the predominant PI 36:1 only reached nominal significance upon repetition of the mass spectroscopic analysis. For this reason, the side chain determination of PI 40:4 and PI 36:1 in this model remains unresolved at this time, however this would be of future interest.
FIGURE 5.8: Regulation of intracellular PI species with PA- and PAMN-treatment in FAO hepatoma cells.

Intracellular phosphatidylinositol levels in vehicle- (black), PA- (grey) or PAMN-(white) treated cells. (a) Total intracellular phosphatidylinositol remain unchanged with palmitate, metformin and sodium salicylate treatment. (b) Individual phosphatidylinositol species with changed levels following treatment with palmitate, metformin and sodium salicylate. (c) Individual phosphatidylinositol species which did not change after treatment with palmitate, metformin and sodium salicylate. Data are expressed as mean ± SEM, n=6, *p<0.05 versus vehicle lipid levels, †p<0.05 versus palmitate lipid levels.
5.3.8.1. **Characterisation of lipid side chains on phosphatidylinositol 38:5**

There is currently little information available indicating whether the identity of the PI signalling lipid is important for its ability to be involved in signal transduction. Therefore, determining the identity of PI 38:5 may for the first time provide the identity of a key signalling lipid. PI 38:5 is of interest as it decreased following PA-treatment, making it a possible candidate for impaired insulin action observed with PA-treatment in these cells - correlating with the regulation of glucose production in this model. The mass and expected negative ion mass/charge ratio (m/z) of the likely species appears in Table 5.2.

<table>
<thead>
<tr>
<th>Expected Fragment</th>
<th>Mass (g/mol)</th>
<th>Mass of negative ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C18:0 (stearic acid)</td>
<td>284.5</td>
<td>283.5</td>
</tr>
<tr>
<td>C18:1 (oleic acid)</td>
<td>282.5</td>
<td>281.5</td>
</tr>
<tr>
<td>C20:4 (arachidonic acid)</td>
<td>304.5</td>
<td>303.5</td>
</tr>
<tr>
<td>Inositol Head Group</td>
<td>242</td>
<td>241</td>
</tr>
</tbody>
</table>

As proof of principle, a de-identified human plasma sample (provided by the Meikle lab, BakerIDI) was analysed. Retention time for the precursor ion was 5.4 validating the identity of the lipid. The main peaks of the product ion scan had m/z of 240.8, 281.2 and 303.4, corresponding to the inositol head group, C18:1 and C20:4 respectively (Table 5.3, **Appendix 6**).
One random sample from each of the vehicle-, PA- and PAMN-treated samples was then subjected to the product ion scan protocol. Retention time for the precursor ions fell within the predicted range (vehicle: 5.41 min; PA: 5.38 min; PAMN: 5.41 min). After fragmentation, the inositol head group was also detected in each sample within the predicted m/z range (240.8 - 241.3 m/z). The predicted side chains of arachidonic acid and oleic acid were detected in each of the samples (Table 5.3, Appendix 6). In addition, smaller peaks at 255.0 m/z and 329.2 m/z were identified in the vehicle-treated sample, which correspond to the mass of C16:2 and C22:3. These side chains also add to 38:5, and may therefore represent a minor PI 38:5 lipid, present at a lower concentration than oleic and arachidonic acid containing species (Appendix 6). The identification of the side chains of PI 38:5 may be the first characterisation of a specific PI species of importance in the insulin signalling cascade. Further experimentation will be required to confirm this finding (see Discussion).
**TABLE 5.3: Identified peaks from the product ion scans of PI 38:5.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mass of negative ion (m/z)</th>
<th>Identified lipid side chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-identified Human plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>281.2</td>
<td>C18:1 (oleic acid)</td>
</tr>
<tr>
<td></td>
<td>303.4</td>
<td>C20:4 (arachidonic acid)</td>
</tr>
<tr>
<td></td>
<td>240.8</td>
<td>Inositol Head Group</td>
</tr>
<tr>
<td>Vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>281.3</td>
<td>C18:1 (oleic acid)</td>
</tr>
<tr>
<td></td>
<td>303.2</td>
<td>C20:4 (arachidonic acid)</td>
</tr>
<tr>
<td></td>
<td>240.8</td>
<td>Inositol Head Group</td>
</tr>
<tr>
<td>PA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>281.1</td>
<td>C18:1 (oleic acid)</td>
</tr>
<tr>
<td></td>
<td>303.1</td>
<td>C20:4 (arachidonic acid)</td>
</tr>
<tr>
<td></td>
<td>240.8</td>
<td>Inositol Head Group</td>
</tr>
<tr>
<td>PAMN</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>281.3</td>
<td>C18:1 (oleic acid)</td>
</tr>
<tr>
<td></td>
<td>303.1</td>
<td>C20:4 (arachidonic acid)</td>
</tr>
<tr>
<td></td>
<td>241.3</td>
<td>Inositol Head Group</td>
</tr>
</tbody>
</table>

5.3.9. Increased lipid levels for species containing palmitate side chains was not common to all measured species

In order to assess how palmitate might be incorporated and metabolised by cells, only those species measured which had at least one PA chain were grouped together and assessed (Fig. 5.9). No changes in lipid levels were observed in any DAG species containing PA following either PA- or PAMN-treatment (Fig. 5.9 a). Given one of the more prevalent current hypotheses of lipid-induced glucose deregulation is via DAG-mediated activation of PKC isoforms (reviewed in [280]), this result highlights the necessity for the metabolism of PA to other lipids in order to up regulate DAG levels. In contrast, there was significant up regulation in the
levels of nine PA-containing TAG species (14:0 16:0 18:2, +224 ± 69%, nominal $p=0.0331$; 14:1 16:0 18:1, +222 ± 38%, nominal $p=0.0061$; 18:1 14:0 16:0, +165 ± 39%, nominal $p=0.0166$; 16:0 16:0 16:0, +114 ± 27%, nominal $p=0.0132$; 16:0 16:0 18:2, +184 ± 47%, nominal $p=0.0263$; 16:0 16:1 18:1, +188 ± 60%, nominal $p=0.0427$; 16:0 16:0 18:0, +177 ± 29%, nominal $p=0.0044$; 16:0 18:1 18:2, +354 ± 107%, nominal $p=0.0320$; 16:0 18:0 18:1, +212 ± 71%, nominal $p=0.0483$), while only four PA-containing TAG species remained unchanged following PA-treatment compared with vehicle-treatment (Fig. 5.9 b). Of the remaining PA-containing lipids, there were four ceramides, one cholesterol ester, one phosphatidylglycerol and one sphingomyelin. None of these species were changed following PA-treatment compared with vehicle-treated cells. No PA-containing lipid species were altered following PAMN-treatment compared with PA-treatment.

These results indicate that the changes observed for all lipid species described above are not only a result of direct PA incorporation, but rather result from widespread regulation of lipid metabolism leading to the conversion of PA to other long chain fatty acids and subsequent downstream fluctuations in global lipid metabolism.
FIGURE 5.9: Regulation of lipid species with PA side chains in PA- and PAMN-treated FAO hepatoma cells.
Intracellular lipid species with palmitate side chains in vehicle- (black), PA- (grey) or PAMN- (white) treated cells. (a) All measured DAG species with PA side chains remain unchanged with PA- or PAMN-treatment. (b) All measured TAG species with PA side chains following PA- and PAMN-treatment. (c) All remaining lipid species with PA side chains following PA- and PAMN-treatment. Data are expressed as mean ± SEM, (a-b) n=3, (c) n=6, *p<0.05 versus vehicle-treated lipid levels, †p<0.05 versus PA-treated lipid levels.
5.3.10. PA-treatment was not associated with changes to the gluconeogenetic genes *Pepck* or *G6Pase*

Finally, to further investigate the changes in glucose production observed in this model, RT-PCR analysis of the gluconeogenetic genes *Pepck* and *G6Pase* was performed. PA-treatment did not significantly alter the gene expression levels of either *Pepck* or *G6Pase* (Fig. 5.10). Although there was a trend for a reduction in *Pepck* expression with PA-treatment this did not reach significance (*p*=0.1120). PAMN-treated cells had significantly lower expression of *Pepck* than vehicle-treated cells (*p*=0.0033), however this was not significant versus PA-treated cells. The lower *Pepck* expression observed here in PAMN-treated cells appears consistent with lower glucose production in these cells. In contrast however, the changes observed in *Pepck* expression with PA-treatment were in conflict with the increase in glucose production, indicating that expression of this gene may not be the predominant regulator of glucose production in this model. The expression of *G6Pase* was not altered with either PA- or PAMN- treatment (Fig. 5.10).
FIGURE 5.10: Regulation of the gluconeogenetic genes *Pepck* and *G6Pase* with PA and PAMN-treatment.

Relative gene expression levels for (a) *Pepck* and (b) *G6Pase* in vehicle- (black), PA- (grey) and PAMN- (white) treated FAO hepatoma cells. Gene expression levels are normalised to cDNA concentration. Data are expressed as mean ± SEM, n=8, *p*<0.05 versus vehicle-treated, †p<0.05 versus PA-treated.
5.4. Discussion

The main hypothesis drawn from the results of Chapter 4 was that the transcriptional alterations in the pathways leading to cholesterol and bile acid metabolism may have impacted upon cellular levels of cholesterol and bile acids, and as such may be responsible for the regulation of glucose production in FAO cells. In this chapter, the levels of both intracellular and excreted cholesterol and bile acids were determined in order to test this hypothesis. There was no detected difference between either vehicle-, PA- or PAMN-treated cells for either total intracellular or excreted cholesterol or bile acids. The only difference observed was for cholesterol esters, which were increased with PA-treatment compared with vehicle-treated cells. Therefore, it is possible that the regulation of this metabolic pathway at the transcriptional level has had an effect on the metabolism of cholesterol ester species. However, it is also true that the increase in cellular lipid content with PA-treatment has flowthrough effects on cellular lipid metabolism as the cellular lipid pool reaches a state of metabolic equilibrium – and therefore the contribution of transcriptional regulation of these pathways is inconclusive at this stage.

Despite the measured steady state of cholesterol and bile acids in this study, no conclusions can be drawn about the metabolic flux through these pathways until further analysis is performed. Due to the comprehensive transcriptional decreases observed in the cholesterol and bile acid metabolic pathways, both the synthesis and degradation of these metabolites could have been affected. Therefore, total levels of cholesterol and bile acids may not change, despite changes in metabolic flux through the pathway. Stable isotope labelling of key intermediates or
precursors of this pathway such as acetyl-CoA and cholesterol, alongside the cellular treatments used in this chapter (vehicle, PA and PAMN) would allow for the rate of flux though these pathways to be determined. This would resolve the unanswered questions remaining with regards to cholesterol and bile acid synthesis. Recently, a conference paper presented by Dr Carsten Schmitz-Peiffer of the Garvan Institute Of Medical Research at the Australian Diabetes Society’s 2012 annual meeting showed similar pathway regulation of acetyl-CoA and cholesterol metabolism in high-fat fed PKCe⁻/⁻ mice compared with high fat fed wild type control mice (the model recently published in [94]). Consistent with the findings of the current study, the presented paper did not observe any changes to intrahepatic total cholesterol levels. Metabolic flux through this pathway was determined, by using ¹⁴C-acetate as a precursor for cholesterol synthesis. PKCe⁻/⁻ liver cells were incubated with ¹⁴C-acetate for 4 – 20 h, followed by Folch extraction of lipids, thin layer chromatography, and exposure to film for radioactive imaging. Altered metabolic flux through this pathway to cholesterol was found in PKCe⁻/⁻ liver cells compared with wild type cells. The same analysis could be performed on PA- and PAMN-treated FAO hepatoma cells in order to determine if the pathway regulation described in Chapter 4 is consistent with an altered metabolic flux through this pathway.

The transcriptional regulation of PI metabolism was described in Chapter 3 in response to PA-treatment. A comprehensive analysis of the PI content of vehicle-, PA- and PAMN-treated cells identified PI 38:5, which decreased with PA-treatment and had a trend (p=0.0691 compared with PA-treated) for an increase following PAMN-treatment. While the metabolism of some PI species and the relevant
enzymes involved have been previously studied [281], the contribution of specific PI species in insulin signalling and hepatic glucose production remains unresolved. For this reason, further study was completed which identified the side chain identity of PI 38:5. Taken together, the product ion scans indicate the major identity of PI 38:5 across the three treatment groups was C18:1 (oleic acid) and C20:4 (arachidonic acid). The reduction in this C18:1 containing species is particularly relevant in the context of DAG metabolism as outlined below, where a general increase in DAG species with C18:1 side chains was identified in response to PA-treatment (Fig. 5.2b). A lower level of the side chains C16:2 (hexadecadienoic acid) and C22:3 (docosatrienoic acid) were identified in vehicle treated cells, indicating a second potential minor identity for this species. The identification of the side chains of PI 38:5 is the first characterisation of a specific PI species of importance in the insulin signalling cascade. Further experimentation will be required to confirm this finding, such as to determine whether the identity of the side chains in PI species affects their ability to be phosphorylated (PIP\textsubscript{2} to PIP\textsubscript{3} phosphorylation) in response to insulin. This could be achieved by utilising commercially available antibodies for PIP\textsubscript{2} and PIP\textsubscript{3}. These antibodies allow for the immunoprecipitation of the PIP\textsubscript{2} and PIP\textsubscript{3} pools from basal and insulin stimulated FAO cells, which could then be extracted and undergo lipidomic analysis as described in this chapter. This would indicate for the first time whether the identity of the side chains of PI species are important in their ability to become phosphorylated in response to insulin. In addition, the same experiment could then be performed on vehicle-, PA- and PAMN-treated cells in the presence and absence of insulin to determine whether this hypothesis is true of the model detailed in this thesis.
Increased levels of DAGs, TAGs, and cholesterol esters were all observed with the induction of insulin resistance and elevated glucose production in the liver cells. The role that these species play in the induction of elevated glucose production in the liver remains unresolved. Mice which lack the microsomal triglyceride transfer protein (Mttp) in the liver (a protein that blocks hepatic VLDL secretion) display steatosis characterised by accumulation of TAG, DAG and ceramide species, while retaining normal glucose homeostasis [282]. However, given that incubating hepatic cells with lipids as in this study, and both high fat feeding in mice [144] and lipid infusions in mice [111] and human patients [283] have been shown to cause increased hepatic glucose production and insulin resistance, it is clear that individual lipid metabolites are capable of inducing insulin resistance and high glucose production in the liver. The identity of such species however remains elusive. By looking for species that were reduced with PAMN-treatment, we propose that we can identify potential mediators of glucose production.

The role of TAGs as a mostly neutral storage pool for lipids makes it unlikely that any of these species is directly involved in the induction of insulin resistance or the dysregulation of glucose production as observed in this model, however metabolism of TAG species towards other more toxic species – such as DAGs – makes the capacity of the TAG storage pool an important element in the development of lipid-induced metabolic disorders.

DAG species are a plausible candidate for this effect due to their role in PKCε activation [284], and subsequent Ser307 phosphorylation of IRS-1 [84], leading to inhibition of downstream signalling. Three DAG species in this study were found to
decrease with PAMN-treatment – 18:1 18:1, 18:0 18:1 and 18:1 20:0. Similarly two
TAG species followed this profile – 18:0 18:1 18:1 and 18:0 18:0 18:1, as well as one
cholesterol ester – 18:1. Sung et al [269] have reported that the ability of lipids to
activate PKC isoforms is proportional to the degree of unsaturation of the lipid
species. A lowering in levels of oleic acid containing lipid species in cells treated
with PAMN may therefore lead to a lowering of glucose production by reducing
metabolically toxic DAG species responsible for PKCε activation. The increase in
DAG incorporation of C18:1 in these cells with PA-treatment follows the decrease in
the C18:1 containing PI 38:5, indicating a potential metabolic shift in the storage of
oleic acid away from this PI species and towards DAG incorporation.

In light of the known effect of DAGs on inducing insulin resistance and elevated
glucose production in the liver, it may at first seem contradictory that the decrease
in glucose production observed with PAMN-treatment was not accompanied by a
restoration of normal TAG and DAG levels. However the main effect of PAMN-
treatment in the current study was to lower basal glucose production, and insulin
resistance remained in the PAMN-treated cells. Therefore, by treating with
metformin and sodium salicylate, we appear to have bypassed the insulin resistant
insult to independently lower glucose production in the face of PA-treatment. The
disconnect between basal (insulin free) and insulin-stimulated (0.1 nM insulin)
glucose production presented here is not completely true of the in vivo condition,
where a basal level of insulin will be present even in the fasted state. However,
decreasing the baseline glucose production (as with metformin treatment) in an in
vivo diabetic state remains an effective therapy against T2D [200].
Early studies performed in the 90s showed *in vivo* increases in total ceramide levels in both the muscle and the liver of obese, insulin resistant Zucker rats compared with lean, control animals [96]. In the intervening years, ceramide accumulation was shown to play a critical role in the development of insulin resistance in muscle [152]. The link between ceramide accumulation and insulin resistance in the liver however has not been as clearly defined, with recent studies reporting hepatic accumulation of ceramide in high fat fed acid sphingomyelinase (*Asm*) knockout mice, despite the mice maintaining the same blood glucose levels during a GTT and ITT as knockout animals on normal chow, and wild type controls on a high fat diet [97] – indicating that the accumulation of hepatic ceramide does not induce insulin resistance in these mice. Recent studies have shown that HepG2 cells possess the ability to actively secrete ceramide in a dose and time dependant manner, despite maintaining relative stability in intracellular levels [100]. This evidence suggests the liver possesses an ability to sense rising intracellular ceramide levels and actively excrete ceramide to preserve a stable intracellular concentration. It is therefore possible that the liver has an ability to excrete potentially harmful ceramide metabolites as a protective mechanism [100]. This hypothesis may explain the relative stability in intracellular ceramide levels in the current study in the face of PA-treatment. Measurement of extracellular ceramide levels would need to be performed in vehicle-, PA- and PAMN-treated conditioned media in order to determine if this mechanism was contributing to the relative stability of intracellular ceramide levels in the current study.
The transcriptional regulation of *Pepck* has been widely regarded as the rate limiting step of *de novo* glucose production by the liver for the past 30 years [285]. *Pepck* has been reported to be overexpressed across multiple rodent models of diabetes where elevated hepatic glucose production is present [286]. Given the multilayered regulatory control of *Pepck* expression [287], and the apparent parallel between *Pepck* expression levels and hepatic glucose output, expression levels of *Pepck* have widely been used as an indicator of hepatic glucose production. Interestingly, the increase in basal glucose production observed with PA-treatment in this study was in the absence of changes in either *Pepck* or *G6Pase* gene expression levels, as was the observed decrease in basal glucose production with the addition of metformin and sodium salicylate. Previous studies in primary rat hepatocytes found that the metformin induced decrease in glucose production occurred with decreased *G6Pase* expression and no change to *Pepck* expression [164] – consistent with the findings of this study for *Pepck* expression but in contrast to the measure *G6Pase* expression. Further, a study by Burgess *et al.* [288] showed that in mice with varying degrees of *Pepck* expression in the liver, a 90% reduction in *Pepck* gene expression only resulted in a 40% decrease to flux through the hepatic glucose production pathway. Therefore, the use of *Pepck* gene expression as a proxy for hepatic glucose production is not always accurate and should be interpreted as a relevant mechanism for elevated glucose production only when the relevant rate of glucose production has been measured.

The metabolic fate of PA in PA-treated cells appears to lie in both direct TAG incorporation (Fig. 4.1 b) as well as metabolism to longer chain and unsaturated species (Fig. 4.10). The reduction in glucose production observed with PAMN-
treatment occurred independently of changes to TAG species with PA side chains, suggesting that these TAG species are not playing a significant role in the regulation of glucose homeostasis. This further adds evidence to the role of non-PA containing species, generated as a consequence of increased PA metabolism, in the regulation of glucose homeostasis in this model.

In Chapter 4 the reversal of high glucose production in the liver was characterised by transcriptional regulation of cholesterol and bile acid biosynthetic pathways. While the total levels of cholesterol and bile acids remained unchanged with PA- and PAMN-treatment, it is possible that metabolic flux through these pathways has been altered, and the emerging role of this system in regulation of glucose metabolism in the liver makes it an attractive target for therapeutic intervention. A detailed profile of lipid metabolites across both PA-treated (insulin resistant, high glucose producing) and PAMN-treated (low glucose producing) cells was also compiled. The identification of multiple lipid species, mostly containing C18:1 side chains, regulated in parallel with a reduction in glucose production indicates potential mediators of both insulin resistance and regulation of glucose production in the liver. Therefore, the main aim for this chapter – utilising a mass spectroscopic based lipidomic analysis of vehicle, PA- and PAMN-treated cells in order to determine whether the changes observed in transcriptional regulation as outlined in Chapter 4 affect the metabolism of lipids in these cells – has been successfully completed. The secondary aim of this chapter – to utilise specific targeted approaches to investigate the metabolism of cholesterol and bile acids – was also successfully completed.
Chapter 6 – Conclusions and Future Directions

The overall aim of this project was to investigate the utility of whole genome microarray techniques to (1) identify candidate GES genes as a new and unbiased screening tool, and (2) to identify metabolic pathway regulation, in a model of lipid-induced dysregulated glucose metabolism.

The findings reported here suggest that the transcriptional changes associated with PA-induced dysregulation of hepatic glucose production are relatively low compared to other models of insulin resistance and dysregulated glucose metabolism [141]. Strong evidence was presented in Chapter 4 that the reduction in hepatic glucose production by metformin and sodium salicylate comprehensively decreased the gene expression levels of all the enzymes regulating the conversion of acetyl-CoA to cholesterol, and the effects of these transcriptional changes on lipid metabolism were described. These changes could not be measured at the level of the metabolite cholesterol using the techniques employed in this study, however this research is ongoing, and will be outlined below. Finally, a novel phosphatidylinositol species PI 38:5 was identified, and along with two other members of the PI family (PI 36:1 and PI 40:4) warrant further investigation as to their contribution to insulin signalling and hepatic glucose production.

This chapter will summarise the findings of this study, and discuss the possible significance in vivo. Future studies (1) to identify candidate GES genes for a PA-induced model of dysregulated glucose production, and (2) to investigate the role of phosphatidylinositol and cholesterol metabolism in the regulation of glucose production, will be discussed.
6.1. Identification of candidate GES genes for confirmation in a human cohort

The DLDA approach utilised in this study identified 14 candidate GES genes. Initial analysis of the expression levels of the seven genes, which were mapped to known genes using BLAST search, could not be verified by RT-PCR analysis. One major reason for lack of correlation between the microarray and RT-PCR analysis was the relatively small fold changes observed for the candidate genes following PA-treatment, reducing the ability to detect differential gene expression [220, 222, 223]. This varies from previous GES attempts by our laboratory where both a larger number of candidate genes, alongside greater fold changes in gene expression levels enabled the successful validation of a five gene GES responsive to the pro-inflammatory cytokine TNFα [141]. Therefore, the identification of additional genes to identify a PA-based GES is necessary if this aspect of the project is to be completed. This could occur by one of two ways – either identify new genes in the current microarray dataset that were not found in the initial DLDA attempt, or identify the unknown genes from the original DLDA attempt.

Firstly, by relaxing the stringency of the DLDA analysis, a greater number of candidate genes may be identified. By expanding the number of candidate genes via this approach, the chance of finding a gene that may be validated by RT-PCR should be increased. Unfortunately, the Acuity software package available to our laboratory cannot be modified to allow for alternative criteria for gene selection. Alternative software packages available to perform this role include GeneSpring (Agilent), however these software packages have a significant recurring cost and as such remain unavailable for use in this project at this time. An alternative approach
could be to exclude all genes with fold changes less than 2.0 from the dataset prior to the DLDA, as this is a commonly suggested limit under which decreased correlation is observed [224]. However, given the fold-changes observed in the top candidate genes identified through DLDA, it is unlikely that there are many genes with greater fold-changes, which are highly significant discriminators of all three treatments. This approach may therefore limit the number of genes in the dataset to a very small subset, thus making any further analysis difficult.

An effective alternative is the use of Bayesian statistical analysis to identify candidate genes. The power of this approach is that the strength of the gene set as a whole in discriminating between the various biological states can be calculated. Although this side of the analysis was completed too late for inclusion in this thesis, the identification of candidate genes via this approach is currently ongoing. Interestingly, it appears at this early stage that many of the same genes are being identified as were described by DLDA in this study – which may somewhat limit this approach for candidate gene identification for this model. The correlation between DLDA and Bayesian gene identification has been previously described by our group [141].

Identification of the seven genes that were unable to be mapped to known genes using a BLAST search may allow for the description of previously unknown genes. Initial experimentation would involve extending the 60mer sequence from the Agilent microarray for each of the unknown candidate genes, which may yield additional information as to the identity of the genes. With additional sequence
length, possible protein sequences could be identified, and *in silico* analysis to predict if the sequence contains any known or relevant domains.

The San Antonio Family Heart Study data [150] is available for further data mining upon identification of alternative putative GES genes. In addition, the use of alternative human cohorts would be sought for further validation of putative GES genes.

### 6.2. The role of Anxa3 in dysregulated glucose production and whole body insulin resistance

Further to the development of a GES with the candidate genes, analysis of the individual genes that were identified via DLDA in the microarray data may provide further insight into the mechanism of action of the PA- and PAMN-treatments observed in this study. *Anxa3* was identified as a candidate GES gene, and was also found to be expressed in human lymphocyte tissue (a pre-requisite for our GES development). Human phenotype data was interrogated to observe if the expression of *Anxa3* was associated with the metabolic syndrome. Despite showing a correlation to fasting blood glucose and total serum triglycerides, this was not independent of age, sex and BMI. Although expression of this gene was not found to be an independent predictor of fasting blood glucose, a role for *Anxa3* in regulation of glucose metabolism has been described through its inhibitory interaction with another of the candidate genes identified in this study – *Pla2g1b*. It is hypothesised that the increase in *Anxa3* gene expression observed with PA-treatment is acting as a cellular defence mechanism against excessive cellular lipid
levels. Following the regulation to lipid metabolism with PAMN-treatment, Anxa3 gene expression levels decrease due to other defences against PA-stress being present (altered lipid metabolism).

To further assess the ability of Anxa3 to regulate hepatic glucose production a number of additional experiments will need to be performed. Both gain- (overexpression) and loss- (knockdown) of-function studies for Anxa3 will be required to answer these questions. Overexpression of a HA-tagged Anxa3 has been achieved using the pcDNA3.1/Zeo plasmid to transfect human embryonic HEK293T kidney cells [289]. In addition, the use of a lactacystin-inducible Anxa3 pT-Rex-DEST30-ANXa3 plasmid has been described in primary cortical neurons from mouse embryos [290]. Knockdown of Anxa3 has been achieved with RNA interference (RNAi) in primary parenchymal rat hepatocytes [291]. The validation of overexpression and knockdown of Anxa3 has been successfully achieved in these studies using RT-PCR (for mRNA) and western blotting (protein levels). In both gain- and loss-of-function conditions, the effects of Anxa3 overexpression or deficiency on glucose production would be assessed. It is proposed that increasing the gene expression of Anxa3 would inhibit a PA-induced increase to glucose production. It is further proposed that in Anxa3-deficient cells, basal glucose production would increase, and PA-induced glucose production would be further increased. Western blotting and/or RT-PCR of key insulin signalling proteins such as IRS-2, AKT, Foxo1, Pepck and G6Pase would identify whether any observed phenotypes act independent to the effects of insulin, as suggested by the results observed in PAMN-treatment FAO cells. In the knockdown experiments, it could be determined whether Anxa3 expression is required for the actions of either PA or metformin and
sodium salicylate on glucose production in FAO cells. The use of an inducible Anxa3 construct (for example, the lactacystin-inducible Anxa3 plasmid described above [290]) would allow for the role of Anxa3 in the induction and/or reversal of PA-dysregulated glucose production to be temporally distinguished, by inducing Anxa3 gene expression alongside either PA- or PAMN-treatment. Finally, if Anxa3 was found to modulate glucose production, further analysis would be performed to determine if the actions of Anxa3 are in fact due to its inhibitory role on Pla2g1b as proposed in Chapter 3.

6.3. The contribution of the cholesterol metabolism pathway to whole body glucose metabolism

Evidence has been presented in Chapter 4 for widespread transcriptional regulation of cholesterol metabolism following PAMN-treatment. Comprehensive decreases in the gene expression levels for all of the members of the metabolic pathways in the terpenoid backbone biosynthesis and steroid biosynthesis pathway linking acetyl-CoA to cholesterol was identified. The transcriptional alterations in these pathways may impact upon the generation and degradation of available cholesterol and bile acids, and as such may be responsible for the regulation of glucose production in FAO cells.

Given the inability to demonstrate altered levels of total cholesterol or bile acid in this study, further analysis is required to confirm the importance of these metabolic pathways in hepatic glucose production. Given the transcriptional regulation described in Chapter 4, it appears that metabolism at both the level of synthesis
and degradation of these metabolites could have been affected. Therefore, it is possible that while total levels of cholesterol and bile acids may not change, metabolic flux through these pathways may be altered. Dr Carsten Schmitz-Peiffer of the Garvan Institute Of Medical Research has established protocol for measuring metabolic flux using \(^{14}\text{C}\)-acetate as a precursor for cholesterol synthesis, which could be adapted for future use in the current study. Briefly, vehicle-, PA- and PAMN-treated FAO hepatoma cells in 6 cm dishes are incubated for 2 h in serum free RPMI medium, and then incubated for 4 – 20 h with and without 1 nM insulin in the presence of 3 \(\mu\text{Ci}\) \(^{14}\text{C}\)-acetate. Cells are then washed well in ice-cold 1 x PBS pH 7.4, scraped into 250 \(\mu\text{L}\) 1 M NaCl, and lipids isolated using a standard Folch extraction. 10 \(\mu\text{L}\) of each sample, and TAG, DAG, cholesterol and cholesterol ester standards, are spotted onto a silica gel TLC plate and resolved using hexane/diethyl ether/acetic acid (80:40:4). Once dry, the TLC plate is exposed to film for 1 – 2 weeks at -80°C, and densitometry is used to calculate the relative incorporation of \(^{14}\text{C}\) into the different lipid species over time.

The identification of the cholesterol biosynthetic pathway discussed in this study has further implications for the importance of wider lipid metabolism in the regulation of de novo glucose production in the liver. The comprehensive down-regulation of this pathway in response to metformin and sodium salicylate identified this pathway as a major regulated metabolic network in the lowering of hepatic glucose production. This finding offers the potential for novel therapeutic targets for the control of elevated hepatic glucose output.
6.4. Confirming the role of PI side chains in the insulin signalling cascade

Evidence identifying the metabolism of PI species as a potential mediator of the PA-induced glucose dysregulation was described in Chapter 4. The contribution of transcriptional regulation of PI metabolism was confirmed by employing mass spectroscopic analysis of PI species in vehicle-, PA- and PAMN-treated cells, which identified three PI species regulated by PA-treatment: PI 38:5, PI 36:1 and PI 40:4. Further analysis revealed the predominant side chain identity of PI 38:5 to be oleic acid (C18:1) and arachidonic acid (C20:4), and similar analysis could be employed to determine the side chain identity of the two other PI species. The contribution of side chain identity to the ability of PI species to undergo phosphorylation and contribute to insulin signalling has not been previously investigated – possibly due to the lack of technological advances employed in this study which enabled the identification of multiple individual lipid species and their side chains. A study by Wang et al. in 2000 used biotinylated PIP3 species with short, water soluble acyl side chains (C4 and C8) as an affinity ligand to determine novel binding partners [292]. The authors note that binding affinity between di-palmitoyl PIP3 (PI with two C16:0 side chains) and the SH2 domain of the p85 subunit of PI3K decreased as the length of the acyl moiety was shortened – however this observation was included as unpublished data. The binding affinity of PIP2 – and thus the ability to transduce insulin-stimulated signalling through this pathway was not mentioned. If this unpublished finding is accurate, then it correlates with the hypothesis that PI side chains affect binding affinity to other ligands, or in other phosphorylation states.
As discussed briefly in Chapter 5, determination of whether the side chain identity of a PI species affected the ability of that PI species to be phosphorylated (PIP2 to PIP3 phosphorylation) is required. Initially, immunoprecipitation using the PIP2 and PIP3 antibodies would allow for isolation of the PIP2 and PIP3 pools following the presence or absence of insulin stimulation of FAO cells. A Folch extraction of these lipid pools would be performed, followed by mass spectroscopic analysis of the side chains in each pool. This would indicate for the first time whether the longer side chain containing species showed an increased ability to undergo insulin-stimulated phosphorylation. To confirm whether this method of regulation plays a role in the model as presented in this thesis, western blotting experiments utilising PIP2 and PIP3 antibodies would identify if a defect at the level of PIP2 to PIP3 phosphorylation does exist following PA-treatment. If this was shown to be the case, the same immunoprecipitation experiment as described above could then be performed on vehicle-, PA- and PAMN-treated cells in the presence and absence of insulin-stimulation to determine whether this hypothesis is true of the model detailed in this thesis.

6.5. Summary

As noted in Chapter 4, pathway regulation observed at the gene expression level between vehicle- and PA-treatment was different to the regulation between PA- and PAMN-treatment. Given this distinction, it is hypothesised that restoring dysregulated pathways induced by PA-treatment offers only a limited scope for discovering new therapeutic interventions. By observing regulated pathways in the reversal of PA-induced dysregulated glucose production (as with PAMN-treatment
in the current study), alternative pathways not observed with PA-alone can be found. The major limitation of this finding is the limited number of vehicle-samples \( n=8 \) compared with PA- and PAMN-samples \( n=20 \), which may have impacted on the regulated pathways identified between vehicle- and PA-treatments, given the subtle fold-changes observed for the most discriminating genes (see Chapter 3). The discovery of additional pathways regulated by PA-treatment would either (i) confirm the distinction between the PA-induced and PAMN-reversed dysregulation to glucose production, or (ii) identify co-regulated pathways between these two states – which would confirm the identified pathways as critical for the development of new targeted therapeutics.

Evidence for altered gene expression levels for the pathways regulating acetyl-CoA metabolism and cholesterol synthesis was described in Chapter 4. Acetyl-CoA accumulation has previously been shown to regulate glucose production through allosteric activation of pyruvate carboxylase [112], while cholesterol depletion has been shown to alter lipid transport through disruption of caveolae lipid rafts and associated inhibition of CD36/FAT-mediated lipid transport [256, 258]. This study is the first (to our knowledge) to implicate both of these metabolites in the reversal of PA-induced dysregulated glucose production, and highlights these metabolic pathways as avenues for targeted therapies against elevated glucose production (Fig. 6.1). Three novel phosphatidylinositol species have been identified in the current study – PI 38:5, PI 36:1 and PI 40:4 – and further experiments to investigate the contribution of side chain identity on the insulin signalling cascade and hepatic glucose production have been outlined.

Proposed regulation of lipid metabolism and hepatic glucose production following PA- (blue) or PAMN-treatment (red). Following PA-treatment, acetyl-CoA levels increase, leading to allosteric activation of pyruvate carboxylase, and therefore an increased rate of glucose production. Downstream generation of cholesterol stabilises lipid transport, allowing for further PA uptake in a positive feedback mechanism. PAMN-treatment reduces the rate of cholesterol synthesis, inhibiting lipid transport and reducing PA uptake – thereby reducing allosteric activation of pyruvate carboxylase and lowering glucose production.
Despite being unable to identify a PA-based GES, the identification of two genes with potential therapeutic potential – Anxa3 and Pla2g1b – validates the approach used in GES development. In addition, the data from the current study has multiple future uses. Firstly, generation of a PA-based GES is ongoing utilising a Bayesian approach to gene identification. Secondly, the current lipidomic dataset allows for the potential development of a lipid signature, discriminating between PA-induced dysregulated glucose production and the restored metabolic state.

The findings detailed in this thesis, and summarised in this chapter, detail novel regulatory processes involved in the reversal of dysregulated glucose metabolism as a consequence of lipid overload. These findings significantly contribute to the current understanding of lipid-induced dysregulated glucose metabolism, and offer new targets for the development of novel therapeutics for the treatment of obesity-driven type 2 diabetes.
APPENDIX 1 – In vitro model development of PA-induced dysregulation of glucose homeostasis in muscle cell lines.

a1.1. Introduction
Appendix 1 outlines the initial experiments conducted to develop an in vitro model of PA-induced dysregulation of glucose homeostasis in a muscle cell line. As described in Chapter 2, the initial aim of this study was to develop an in vitro cellular model of PA-induced insulin resistance, followed by the restoration of the impairment in insulin action by trialling known anti-diabetic and insulin sensitising agents. This appendix will therefore outline the different muscle cell lines (C2C12, L6 and L6 stably overexpressing a haemaglutinin epitope on an exofacial loop of GLUT4), and differing insulin signalling endpoints measured (glycogen synthesis, 2-DOG uptake, and HA-GLUT4 translocation, respectively), in the attempt to replicate previously described models of PA-induced insulin-resistance.
a1.2. Methods

a1.2.1. Cell culture

C2C12 mouse myoblasts [293] were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM, 5.56 mM D-glucose, GIBCO Invitrogen, Melbourne, VIC, Australia) supplemented with 10% (v/v) Foetal Bovine Serum (FBS, GIBCO Invitrogen) at 37°C and 5% CO₂. After reaching 100% confluence, C2C12 myoblasts were cultured in low glucose DMEM supplemented with 2% Horse Serum (HS, GIBCO Invitrogen) at 37°C and 5% CO₂ to induce cell cycle withdrawal and differentiation into myotubes [294]. Cells were allowed to differentiate for 5 days before treatment began. Media was replenished every 24 h.

L6 rat myoblasts [295] were cultured in high glucose DMEM (25mM D-glucose, GIBCO Invitrogen) supplemented with 10% (v/v) FBS at 37°C and 5% CO₂. After reaching 100% confluence, L6 myoblasts were cultured in high glucose DMEM supplemented with 2% HS at 37°C and 5% CO₂ to induce differentiation into myotubes. Treatment commenced after 3 days differentiation. Media was replenished every 24 h. L6 myoblasts stably overexpressing a haemaglutinin epitope on an exofacial loop of GLUT4 (HA-GLUT4-L6 cell line kindly provided by Dr Cordula Hohnen-Behrens and Prof David James, Garvan Institute, Sydney, NSW, Australia, [296]) were cultured in either low-glucose DMEM or alpha-MEM (GIBCO Invitrogen) supplemented with 10% (v/v) FBS at 37°C and 5% CO₂. Media was replenished every 24 h.
a1.2.2. **Cell treatments**

C2C12 and L6 myotubes were treated with vehicle (pH 7.4, 0.0285% (v/v) Ethanol (EtOH), 25 μM Bovine Serum Albumin, BSA Albumin, Fraction V, USB Corporation, OH, USA) or 0.05-0.3 mM PA (PA, pH 7.4, dissolved in 0.0285% EtOH and 25 μM BSA) for 24 or 48 h. HA-GLUT4-L6 myoblasts were treated for 16 h with vehicle (pH 7.4, 0.7% (v/v) EtOH, 5% wt/vol BSA) or 0.3-0.75 mM PA (dissolved in 0.7% (v/v) EtOH and 5% wt/vol BSA). The proposed treatment regime for the final model is described in Fig. a1.1.

a1.2.3. **Glycogen synthesis measurement in C2C12 myotubes**

Glycogen synthesis was measured as described previously [152]. PA-treated myotubes in 24-well plates were incubated for 2 h in 200 μL serum-free DMEM containing [U-14C]-D-glucose (1 mCi/ml) in the absence or presence of 100 nM insulin and 0.05-0.3 mM PA. Cells were washed twice in 200 μL of ice-cold 1 x PBS pH 7.4, and then lysed in 300 μL 1 M KOH. Cell lysate was transferred to 1.5 mL eppendorf tube, and 5 μL of 25 mg/mL glycogen in sterile filtered H2O added to each tube. Sample extract was then incubated for 10 min at 100°C. 50 μL of saturated Na2SO4 solution was added to each tube followed by 950 μL of 100% v/v ice-cold EtOH to induce glycogen precipitation. Samples were vortexed and incubated at -80°C for 30 min. Samples were centrifuged at 18,000 x g for 10 min, and supernatant aspirated. Pellets were washed by resuspension in 100 μL H2O, followed by sonication at 50 Hz for 2 min in an Unisonics FXP 8M Sonicator Waterbath (Unisonics, Sydney, Australia) until the pellet had dissolved. 900 μL of 100% v/v EtOH was added to each sample, which were then vortexed and incubated.
at -80°C for 30 min. Samples were centrifuged at 18,000 x g for 10 min, followed by aspiration of the supernatant. The pellet was then dissolved in 100 μL of water and sonicated once more to fully dissolve the precipitate. Samples were mixed with 1.2 mL of scintillate (Ultima Gold, PerkinElmer Life Sciences, Waltham, MA, USA) and counted for radioactivity. Counts per min were measured by a TriCarb 2900TR-LSA scintillation counter (PerkinElmer Life Sciences).

a1.2.4. 2-Deoxyglucose uptake in L6 myotubes

Glucose transport was measured as 2-deoxyglucose uptake (2-DOG) as described previously [297]. Treated L6 myotubes in 24-well plates were washed twice in PBS (GIBCO Invitrogen) containing 0.2% (w/v) RIA-grade BSA, 0.5 mM MgCl₂, and 0.9 mM CaCl₂. Insulin (Humulin R; Novo Nordisk, Baulkham Hills, Australia) at 0 or 100 nM was added for 30 min at 37°C. Uptake of 50 μM 2-DOG and 0.5 μCi 2-deoxy-[U-3H]glucose (NEN, PerkinElmer Life Sciences, Melbourne, Australia) per well was measured over the final 10 min of insulin-stimulation. The reaction was stopped by addition of 200 μL of ice cold 1 x PBS pH7.4 containing 80 μg/ml phloretin (Sigma-Aldrich), and cells were then solubilised in 0.03% (w/v) SDS (GIBCO Invitrogen). Counts per min were measured by a 2900TR-LSA scintillation counter.

a1.2.5. HA-GLUT4 translocation measurement in stable L6-GLUT4 myoblasts

Translocation of GLUT4 to the plasma membrane was measured as previously described [298]. L6 myoblasts that stably overexpress HA tagged-GLUT4 (HA-GLUT4) were seeded into gelatin-coated black-well with clear-bottom 96-well plates.
Optimisation of the translocation assay was performed to determine duration (5 – 30 min) and concentration (1 – 1000 nM insulin) of insulin-stimulation, as well as the serum starvation conditions. The serum starvation buffers tested were 2 h incubation with Krebs Ringer Phosphate buffer (KRP, pH 7.4, 12.5 mM HEPES, 120 mM NaCl, 6 mM KCl, 1.2 mM MgSO₄, 1 mM CaCl₂, 0.4 mM NaH₂PO₄, 0.6 mM Na₂HPO₄, 0.2% BSA w/v) or alpha-MEM media, pH 7.4 containing 0.2% w/v BSA and 20 mM HEPES, or an overnight incubation with bicarbonate free alpha-MEM media, pH 7.4 containing 0.1% w/v FCS. This was followed by the addition of insulin (1 – 1000 nM) over 5 – 30 min at 37°C. The maximum insulin-stimulated HA-GLUT4 translocation and minimal basal HA-GLUT4 translocation was achieved at 200 nM insulin for 20 min at 37°C. Serum starvation for 2 h in alpha-MEM gave the greatest insulin stimulatory response for HA-GLUT4 translocation.

Following serum starvation and insulin-stimulation, cells were then fixed with 3% (w/v) paraformaldehyde on ice for 15 min and for a further 30 min at 22°C and quenched with 50 mM glycine for 5 min at 22°C. Cells were washed twice with 1 x PBS pH 7.4 and then blocked in the absence of 0.1% (w/v) saponin (to measure HA-GLUT4 at the cell surface) or presence of saponin (to measure total cellular HA-GLUT4). The primary antibody, anti-HA.11-purified monoclonal antibody (2 μg/ml; Covance, Vienna), or mouse IgG1κ MOPC21 as a control non-relevant isotype-specific antibody (2 μg/ml; Sigma) in PBS containing 2% (v/v) normal swine serum (DakoCytomaton, Glostrop, Denmark) was added for 45 min at 22°C. The cells were washed 3 times with PBS and blocked again in the presence of saponin (to permeabilise all cells so that the background labelling of secondary antibody is similar for all cells). Cells were incubated for 45 min at 22°C away from light with the secondary antibody (20 μg/ml Alexa 488-conjugated goat anti-mouse
fluorescein isothiocyanate antibody; Molecular Probes) to detect anti-HA.11 antibody. Cells were washed four times with PBS, the fluorescence intensity (excitation 485 nm, emission 520 nm) was measured with the bottom reading mode in a Fusion A1536 fluorescence plate reader (Packard Bioscience, Melbourne, Australia) using Fusion Instrument Control MFC Application software (Packard Bioscience). The percentage of HA-tagged GLUT4 at the cell surface was calculated by dividing the mean anti-HA fluorescence units for each treatment in the absence of saponin (minus non-specific anti-MOPC fluorescence units) by the mean anti-HA fluorescence units in the presence of saponin (minus non-specific anti-MOPC fluorescence units) multiplied by 100.

**a1.2.6. Statistical analysis**

Statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) version 20.0 (IBM). Data were first analysed for normality using a one-sample Kolmogorov-Smirnov test. All data was determined to be normally distributed. Data were then either analysed using independent samples Student’s T-test, or a one-way ANOVA. Homogeneity of variance was determined using Levene’s Test, and post-hoc analysis of ANOVA used either Fisher’s least significant difference (LSD, for homogeneous variance) or Games-Howell (for non-homogeneous variance). Data were considered significant at $p<0.05$. Data are presented as mean ± SEM unless otherwise stated.
FIGURE a1.1: Schematic representation of the 48 h treatment regime for the induction and reversal of PA-induced dysregulation of insulin sensitivity.

a1.3. Results

a1.3.1. Effects of PA-treatment on basal and insulin-stimulated glycogen synthesis in C2C12 myotubes

The initial attempt to model hyperlipidemia-induced “insulin resistance” in vitro used C2C12 myotubes with the incorporation of [U-14C]-D-glucose into glycogen over 2 h as an endpoint measurement of insulin action. It has previously been shown in this cell line [152] that 0.75 mM PA-treatment overnight (16 h) caused approximately 50% decrease in insulin- (100 mM) stimulated (100 nM) glycogen synthesis – completely ablating the effects of insulin-stimulation on glycogen synthesis. Following 24 h incubation with vehicle, 100 nM insulin-stimulated glycogen synthesis was increased by 40 ± 5% versus basal glycogen synthesis (*p*=0.0006). Following 24 h treatment with 0.05, 0.1, 0.2 or 0.3 mM PA, no significant difference was found between any treatment groups and vehicle-treated cells for either basal or insulin-stimulated glycogen synthesis (Fig. a1.2 a), indicating that no defect to glycogen synthesis was observed with PA-treatment at 24 h. Treatment with PA and vehicle was then performed for 48 h. After 48 h treatment with vehicle, insulin-stimulated glycogen synthesis was not significantly different to basal glycogen synthesis (Fig. a1.2 b). Treatment for 48 h with 0.1 mM PA did not significantly change either basal or insulin-stimulated glycogen synthesis compared to the corresponding vehicle-treated cells (*p*=0.571 and *p*=0.897 respectively).
FIGURE a1.2: Inhibition of glycogen synthesis by PA in C2C12 myotubes. 
(a) C2C12 myotubes were treated with 0.05 – 0.3 mM PA for 24 h. Basal (black bars) and 100 nM insulin-stimulated (white bars) glycogen synthesis was measured (passage 13, day 7 post-differentiation). Glycogen levels synthesised from glucose in vehicle-treated, basal cells was equal to 4.70 ± 0.19 nM glucose converted to glycogen. 
(b) C2C12 myotubes were treated with 0.05 – 0.3 mM PA for 48 h. Basal (black bars) and 100 nM insulin-stimulated (white bars) glycogen synthesis was measured (passage 13, day 7 post differentiation). Glycogen levels synthesised from glucose in vehicle-treated, basal cells was equal to 3.41 ± 0.08 nM glucose converted to glycogen. Data expressed as mean ± SEM. *p<0.05 when compared to basal, vehicle-treated cells. †p<0.05 when compared to insulin-stimulated vehicle-treated cells. Each experiment was performed once in triplicate.
Following 48 h treatment with 0.2 mM PA, basal glycogen synthesis was lowered by 70 ± 7% compared with vehicle-treated cells ($p=0.0001$). Treatment with 0.3 mM PA for 48 h decreased both basal glycogen synthesis by 64 ± 2% ($p=0.0003$ versus basal vehicle-treated cells) and insulin-stimulated glycogen synthesis by 70 ± 6% ($p=0.0001$ versus insulin-stimulated vehicle-treated cells, Fig. a1.2 b). While impaired insulin-stimulated glycogen synthesis was observed following 0.3 mM PA-treatment for 48 h, this was preceded by a decrease in basal glycogen synthesis, which was also observed with 0.2 mM PA. Treatment with 0.3 mM PA also lead to a visible decrease in cell number (light microscopic observation), although whether this was due to decreased growth rate, or an increase in cellular death (or both) was not measured. These observations, coupled with the decreased basal glycogen synthesis indicate that PA-treatment at 0.3 mM might be impacting cellular viability as well as insulin sensitivity. For these reasons modelling cellular “insulin resistance” using glycogen synthesis in C2C12 cells was not further utilised.

a1.3.2. Effects of PA-treatment on basal and insulin-stimulated glucose uptake in L6 myotubes

The second model used to measure PA-induced impairment to insulin action was glucose uptake in L6 myotubes. Glucose uptake was not undertaken in C2C12 myotubes due to a poor-response to insulin when measuring glucose uptake [152]. The ability of to induce “insulin resistance” in L6 myotubes was assessed by measuring the uptake of 2-deoxy-[U-$^{14}$C]-glucose (2-DOG) following insulin-stimulation (100 nM, 20 min). Insulin had no effect on 2-DOG uptake in either vehicle-treated cells, ($p=0.9721$ compared to basal cells), nor PA-treated cells (0.5 – 3 mM),
making the determination of PA-induced impairment of insulin action difficult to determine. After 24 h treatment with 0.05, 0.1 or 0.2 mM PA, there was no significant difference to either basal or insulin-stimulated 2-DOG uptake compared with the corresponding vehicle-treated cells (Fig. a1.3 a). Treatment with 0.3 mM PA for 24 h caused a 48 ± 13% decrease in basal 2-DOG uptake ($p=0.0011$) and a 52 ± 6% decrease in insulin-stimulated 2-DOG uptake ($p=0.0004$, Fig. a1.3 a) compared with the respective vehicle-treated cells.

After vehicle-treatment for 48 h, insulin-stimulated 2-DOG uptake was increased by 9 ± 1% in vehicle-treated cells ($p=0.0092$, fig a1.3, b), indicating that the cells were minimally responsive to insulin. Following 0.1 mM PA-treatment, basal 2-DOG uptake was lowered by 13 ± 2% ($p=0.0002$) compared to vehicle-treated basal cells, while the insulin-stimulated effect on 2-DOG uptake was completely ablated ($p=0.5904$ compared to basal 0.1 mM PA-treated cells). Treatment at 48 h with 0.2 mM PA decreased 2-DOG uptake by 18 ± 1% ($p=0.000001$) compared to vehicle-treated basal cells, and maintained the ablated response to insulin. Similarly, treatment with 0.3 mM PA for 48 h decreased glucose uptake by 39 ± 3% ($p=2.1\times10^{-15}$), while insulin was ineffective at altering glucose uptake (Fig. a1.3 b). These cells exhibited a lack of insulin sensitivity at both 24 and 48 h of vehicle- or PA-treatment, and as such building an in vitro model of cellular insulin resistance due to PA was not possible. Numerous passages of L6 myoblasts from a range of different frozen cell stocks were used and all showed similar results (data not shown). During differentiation, the L6 cells were observed to lift from the tissue culture plate surface following 3 days of differentiation, and as such, PA incubations had to begin prior to full differentiation of the cells, in order for the subsequent insulin bioassay to be performed. The lack of significant
insulin response in vehicle-treated cells may therefore in part be explained by the use of incomplete differentiated myotubes possessing an immature insulin response. Using 2-DOG uptake in L6 myotubes to model PA-induced impairment of insulin action was therefore unsuccessful, and was not utilised for further experimentation.
FIGURE a1.3: Inhibition of glucose uptake by PA-treatment in L6 myotubes.

(a) L6 myotubes were treated with 0.05 – 0.3 mM PA for 24 h. Basal (black bars) and 2 h insulin-stimulated (100 nM, white bars) 2-DOG uptake was then measured. Vehicle-treated, basal 2-DOG uptake was 35 ± 2 pmol 2-DOG transported/min/well.

(b) L6 myotubes were treated with 0.1 – 0.3 mM PA for 48 h. Basal (black bars) and 2 h insulin-stimulated (100 nM, white bars) 2-DOG uptake was measured. Vehicle-treated, basal 2-DOG uptake was 32 ± 1 pmol 2-DOG transported/min/well. Data expressed as mean ± SEM. *p<0.05 when compared to basal, vehicle-treated cells. †p<0.05 when compared to insulin-stimulated vehicle-treated cells. Each experiment was performed twice, in triplicate.
a1.3.3. Effects of PA-treatment on basal and insulin-stimulated HA-GLUT4 translocation in stable HA-GLUT4-L6 myoblasts

The final muscle cell-based model for PA-induced insulin resistance measured was translocation of the exogenous HA-tagged GLUT4 to the plasma membrane as a measure of insulin action. This protocol was optimised prior to experimentation (see Methods). The optimum conditions to follow PA-treatment were determined to be serum starvation for 2 h in bicarbonate-free alpha-MEM with 0.2% BSA, followed by 200 nM insulin-stimulation for 20 min at 37°C. A similar protocol which differed only in the timing of serum starvation (16 h) was also used for experimentation, which gave similar results (data not shown).

Following 16 h vehicle-treatment, insulin-stimulation (200 nM, 20 min) increased the amount of HA-GLUT4 at the plasma membrane by 15 ± 1% (of total HA-GLUT4) in vehicle-treated cells (p=0.0002 versus basal vehicle-treated, Fig. a1.4). Treatment with 0.3 mM PA for 16 h did not affect basal HA-GLUT4 translocation, however, insulin-stimulated HA-GLUT4 was decreased by 7 ± 1% (p=0.0363 versus insulin-stimulated vehicle-treated), remaining elevated over basal 0.3 mM PA-treated cells (p=0.0087). Treatment with 0.75 mM PA reduced basal HA-GLUT4 at the plasma membrane by 12 ± 2% (p=0.0016 versus basal 0.3 mM PA-treated) and further reduced insulin-stimulated HA-GLUT4 by 19 ± 1% (p=1.6x10^-5 versus insulin-stimulated 0.3 mM PA-treated, Fig. a1.4). Overall, a minimal effect on insulin action was observed with 0.3 mM PA, while treatment with 0.75 mM PA completely ablated the insulin response as well as lowering basal HA-GLUT4 translocation.
FIGURE a1.4: PA-induced inhibition of HA-GLUT4 translocation to the plasma membrane in stable infected HA-GLUT4-L6 cells.
Effects of 0.3 and 0.75 mM PA-treatment for 16 h on HA-GLUT4 translocation. Cells were serum starved for 2 h in bicarbonate free alpha-MEM with 0.2% BSA, then incubated for 20 min in the absence (black bars) or presence (white bars) of 200 nM insulin at 37°C prior to performing the HA-GLUT4 translocation assay. Data expressed as mean ± SEM. *p<0.05 when compared to basal, vehicle-treated cells. †p<0.05 when compared to insulin-stimulated vehicle-treated cells. ††p<0.05 when compared to 0.3 mM PA-treated, insulin-stimulated cells. Each experiment was performed three times, in triplicate.
The dynamic range of the responses to both insulin and PA were too narrow for further manipulations with known anti-diabetes compounds. In addition, the low percentage of HA-GLUT4 translocation observed in basal treated cells during optimisation of the protocol (26 ± 1%) was not observed in vehicle- (54 ± 1%) or PA- (54 ± 2% and 42 ± 2%) treated cells. The effects of ethanol (from the vehicle-treatment) may in part explain this finding (see Discussion). Due to the narrow dynamic range and high basal HA-GLUT4 translocation to the plasma membrane, modelling HA-GLUT4 translocation as a measure of PA-induced impaired insulin action was not utilised for further study.

An alternative cellular model - an insulin-responsive liver cell line (rat hepatoma FAO, see Chapter 2) [42] was used to model impairment of insulin action due to PA, rather than in vitro muscle cell lines.
a1.4. Discussion

The three muscle cell based models of insulin action detailed in this chapter were unable to demonstrate PA-induced insulin resistance. The effect of PA on glycogen synthesis in C2C12 cells was mainly on basal synthesis, and only affected insulin-stimulated glycogen synthesis at 0.3 mM, at which basal glycogen synthesis was less than half that of vehicle-treated cells (Fig. a1.2). The large reduction in basal measurements indicated that the effects of PA in these cells may have been negatively impacting cellular viability. The second muscle cell model described was 2-DOG uptake in L6 myotubes. At both 24 and 48 h treatment, the largest significant insulin response in vehicle-treated cells was 9 ± 1% (Fig. a1.3). Given this lack of insulin response, these cells were not suitable for use in modelling insulin resistance. Likewise, in HA-GLUT4 overexpressing L6 cells, the response to insulin-stimulation was a 15% increase in HA-GLUT4 translocation to the plasma membrane. This was also coupled with constitutive elevated basal HA-GLUT4 at the plasma membrane, which was higher than observed while optimising the protocol (26 ± 1%). One explanation for the lack of effect may be the effects of EtOH (in the vehicle) on GLUT4 translocation. It has been reported that ethanol at similar concentrations to the vehicle (120 mM) has been shown to modulate glucose uptake in primary cultured rat skeletal muscle cells [299], as well as increase translocation of GLUT4 to the plasma membrane [300], which may explain not only this difference but also the lack of a significant insulin stimulatory effect in both the glycogen synthesis and 2-DOG uptake models as well.

Both glycogen synthesis in C2C12 myotubes and glucose uptake in L6 myotubes were not insulin sensitive in the experiments performed in this study. While these models have been shown to be insulin sensitive in previous research, with insulin-
stimulated responses of 2 fold in C2C12 myotubes [152] and 2.5 fold in L6 myotubes [301] these results could not be replicated. The inability to reproduce a consistent response to insulin in these cells makes them unsuitable for the development of the model for the purposes of this study. Despite this conclusion however the ability of these models to characterise signalling defects underlying lipid-induced insulin resistance remains a valid though limiting utility of the models.
APPENDIX 2 – Reversal of 48 h PA-treatment induced high glucose production in FAO hepatoma cells by combination treatment using metformin and sodium salicylate.

(a) Glucose Production (% of Vehicle)

(b) MTT Cleavage (% of Vehicle)
FIGURE a2.1: Combination reversal of PA-induced glucose dysregulation using 0.1 mM metformin and 2 – 3 mM sodium salicylate in FAO hepatoma cells.

(a) Basal (black bars) and 0.01 nM insulin-stimulated (white bars) glucose production following 48 h treatment with PA, with metformin and sodium salicylate added in the final 24 h of treatment. Vehicle-treated, basal glucose production was equal to 31.44 ± 1.58 μg glucose / mg protein. (b) Effect of PA, metformin and sodium salicylate on cellular viability after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. (c) Effects of PA metformin and sodium salicylate on LDH ratio after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. Average LDH ratio (extracellular:intracellular) for basal, vehicle-treated cells was 0.147 ± 0.010. Data expressed as mean ± SEM. *p<0.05 for basal PA-treated cells when compared to basal vehicle-treated cells. †p<0.05 for basal PA, metformin and/or sodium salicylate treated cells when compared to basal PA-treated cells. ††p<0.05 for insulin-stimulated cells when compared to each individual treatments basal treated cells. M p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant metformin alone treated cells. N p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant sodium salicylate alone treated cells. Each experiment was performed three times, in triplicate.
FIGURE a2.2: Combination reversal of PA-induced glucose dysregulation using 0.25 mM metformin and 1 - 5 mM sodium salicylate in FAO hepatoma cells.

(a) Basal (black bars) and 0.01 nM insulin-stimulated (white bars) glucose production following 48 h treatment with PA, with metformin and sodium salicylate added in the final 24 h of treatment. Vehicle-treated, basal glucose production was equal to 35.03 ± 2.01 μg glucose / mg protein. (b) Effect of PA, metformin and sodium salicylate on cellular viability after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. (c) Effects of PA metformin and sodium salicylate on LDH ratio after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. Average LDH ratio (extracellular:intracellular) for basal, vehicle-treated cells was 0.134 ± 0.007. Data expressed as mean ± SEM *p<0.05 for basal PA-treated cells when compared to basal vehicle-treated cells. †p<0.05 for basal PA, metformin and/or sodium salicylate treated cells when compared to basal PA-treated cells. ††p<0.05 for insulin-stimulated cells when compared to each individual treatments basal treated cells. M p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant metformin alone treated cells. N p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant sodium salicylate alone treated cells. Each experiment was performed three times, in triplicate.
FIGURE a2.3: Combination reversal of PA-induced glucose dysregulation using 0.5 mM metformin and 3 – 5 mM sodium salicylate in FAO hepatoma cells.

(a) Basal (black bars) and 0.01 nM insulin-stimulated (white bars) glucose production following 48 h treatment with PA, with metformin and sodium salicylate added in the final 24 h of treatment. Vehicle-treated, basal glucose production was equal to 37.91 ± 2.66 μg glucose / mg protein. (b) Effect of PA, metformin and sodium salicylate on cellular viability after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. (c) Effects of PA metformin and sodium salicylate on LDH ratio after 48 h treatment, with metformin and sodium salicylate added in the final 24 h of treatment. Average LDH ratio (extracellular:intracellular) for basal, vehicle-treated cells was 0.122 ± 0.002. Data expressed as mean ± SEM. *p<0.05 for basal PA-treated cells when compared to basal vehicle-treated cells. †p<0.05 for basal PA, metformin and/or sodium salicylate treated cells when compared to basal PA-treated cells. ††p<0.05 for insulin-stimulated cells when compared to each individual treatments basal treated cells. M p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant metformin alone treated cells. N p<0.05 for basal or insulin-stimulated PA with metformin and sodium salicylate treated cells when compared to relevant sodium salicylate alone treated cells. Each experiment was performed three times, in triplicate.
### APPENDIX 3 – Genes identified through DLDA.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Reference Number</th>
<th>Agilent ProbeID</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxa3</td>
<td>ref</td>
<td>NM_012823</td>
<td>A_43_P11577</td>
</tr>
<tr>
<td>Anxa3</td>
<td>ref</td>
<td>NM_012823</td>
<td>A_44_P301936</td>
</tr>
<tr>
<td>Anxa3</td>
<td>ref</td>
<td>NM_012823</td>
<td>A_44_P325508</td>
</tr>
<tr>
<td>Gstm3</td>
<td>ref</td>
<td>NM_020540</td>
<td>A_44_P391068</td>
</tr>
<tr>
<td>Pla2g1b</td>
<td>ref</td>
<td>NM_031585</td>
<td>A_43_P15319</td>
</tr>
<tr>
<td>Rab33a</td>
<td>ref</td>
<td>NM_001108257</td>
<td>A_42_P522374</td>
</tr>
<tr>
<td>RGD1560824_predicted</td>
<td>ref</td>
<td>XR_007343</td>
<td>A_44_P401035_8232</td>
</tr>
<tr>
<td>Serpina5</td>
<td>ref</td>
<td>NM_022957</td>
<td>A_42_P759524</td>
</tr>
<tr>
<td>Sla2a2</td>
<td>ref</td>
<td>NM_012879</td>
<td>A_44_P488355</td>
</tr>
<tr>
<td>Sla2a2</td>
<td>ref</td>
<td>NM_012879</td>
<td>A_44_P1256612</td>
</tr>
<tr>
<td>Snn</td>
<td>ref</td>
<td>NM_0010340838</td>
<td>A_44_P255109</td>
</tr>
<tr>
<td>AA998612</td>
<td>tc</td>
<td>TC590201</td>
<td>A_42_P591516</td>
</tr>
<tr>
<td>DY472907</td>
<td>gb</td>
<td>FQ217834</td>
<td>A_44_P329389</td>
</tr>
<tr>
<td>TC603506</td>
<td>tc</td>
<td>TC603506</td>
<td>A_44_PB37764</td>
</tr>
<tr>
<td>TC612212</td>
<td>tc</td>
<td>TC612212</td>
<td>A_44_P626893</td>
</tr>
<tr>
<td>TC628671</td>
<td>tc</td>
<td>TC628671</td>
<td>A_44_P577880</td>
</tr>
<tr>
<td>TC641785</td>
<td>tc</td>
<td>TC641785</td>
<td>A_44_P610017</td>
</tr>
</tbody>
</table>

Anxa3, annexin a3/LC3, lipocortin; Slc2a2, solute carrier family 2 (facilitated glucose transporter), member 2/GLUT2, Glucose transporter 2; Gstm3, glutathione S-transferase mu 3; Pla2g1b, phospholipase A2, group IB; Rab33a, Rab33A, member RAS oncogene family; Serpina5, serpin peptidase inhibitor, clade A, member 5; Snn, stannin.
APPENDIX 4 – KEGG pathways identified through GSEA as being differentially regulated between vehicle- and PA-treated cells.

FIGURE a4.1: Cell cycle pathway.
FIGURE a4.2: Oocyte meiosis.

Prophase I

Cdc27
-33 ± 5% (p=0.0065)

Progesterone

Meiosis I

Meiosis I progression, bipolar spindle formation

Ywhab
-22 ± 4% (p=0.0293)

Sik
-31 ± 6% (p=0.0373)

Meiosis I exit

Cdc27
-33 ± 5% (p=0.0065)

Interkinesis

Meiosis II arrest

Mad2l1
-29 ± 6% (p=0.0495)

Meiosis II

Meiosis II exit

Cdc27
-33 ± 5% (p=0.0065)

Spoll
-32 ± 4% (p=0.0397)

Ccned2
-22 ± 5% (p=0.0391)
FIGURE a4.3: Toll-like receptor signalling pathway.

Ligands: Lipoproteins Peptidoglycans

\[ \text{Tir1/6} \quad \text{(no change)} \]
\[ \text{Tir2} \quad \text{(no change)} \]
\[ \text{Fos} \quad +124 \pm 52\% \ (p=0.0389) \]
\[ \text{Jun} \quad +119 \pm 27\% \ (p=0.0007) \]
\[ \text{Induces transcription of Inflammatory cytokines} \]
\[ \text{Ccl5} \quad -43 \pm 5\% \ (p=0.017) \]
\[ \text{Nfkb} \quad \text{(no change)} \]
\[ \text{Chemotactic effects} \quad \text{(Neutrophils, NK cells)} \]

Ligands: Viral RNAs

\[ \text{Endosomal Tir3/9/7/8} \quad \text{(no change)} \]
\[ \text{Induces transcription of Inflammatory cytokines} \]
\[ \text{Cctc10} \quad -57 \pm 3\% \ (p=0.0021) \]
\[ \text{Chemotactic effects} \quad \text{(T-cells)} \]

Ligands: IFN-β

\[ \text{JAK-STAT signalling pathway} \quad \text{(no change)} \]

FIGURE a4.4: Renal cell carcinoma.

Epithelial cell of a proximal renal tubule

Hepatocyte growth factor

\[ \text{Met proto-oncogene} \quad \text{(no change)} \]
\[ \text{Ptpn11} \quad -26 \pm 5\% \ (p=0.0178) \]
\[ \text{Rapgef1} \quad -32 \pm 5\% \ (p=0.0106) \]
\[ \text{Pik3r3} \quad -35 \pm 3\% \ (p=0.0382) \]
\[ \text{Jun} \quad +119 \pm 27\% \ (p=0.0007) \]
\[ \text{Proliferation} \quad \text{Cell-cycle progression} \]
\[ \text{Cell-junction} \quad \text{Migration} \quad \text{Invasion} \]
\[ \text{Akt} \quad \text{(no change)} \]
\[ \text{Survival} \]
FIGURE a4.5: Leukocyte transendothelial migration.

Within migrating leukocytes → Cell motility
- Directional sensing

Within endothelium

NADPH oxidase (no change)

$\text{Pi3k} - 35 \pm 3\% (p=0.0382)$

$\text{Actn3} +21 \pm 6\% (p=0.0073)$ → Docking structure

Regulation of actin cytoskeleton

$\text{Arhgap5} -29 \pm 7\% (p=0.0450)$

$\text{Ctnnb1} -21 \pm 5\% (p=0.0404)$ → Regulation of

Cell Adhesion Molecules

Transendothelial migration

$\text{Ptpn11} -25 \pm 5\% (p=0.0178)$

$\text{H}_{2}\text{O}_{2}$

FIGURE a4.6: Colorectal cancer.

MAPK signalling pathway → $\text{Kras}$ (no change)

$\text{MAPK1/3}$ (no change)

$\text{Fos} +124 \pm 52\% (p=0.0389)$

$\text{Jun} +115 \pm 27\% (p=0.0007)$ → Induces transcription of

CyclinD1 and promotes proliferation

Wnt signalling pathway → $\text{Pi3k} - 35 \pm 3\% (p=0.0382)$

$\text{Akt}$ (no change)

$\text{Ctnnb1} -21 \pm 5\% (p=0.0404)$ → Regulates transcriptional control of

Anti-apoptosis and proliferative genes
FIGURE a4.7: Neutrophin signalling pathway.

MAPK signalling pathway

- Nerve growth factors
  - Rapgef1: -32 ± 5% (p = 0.0106)
  - Ptpn11: -26 ± 5% (p = 0.0178)
  - Pik3r3: -35 ± 3% (p = 0.0382)

- Akt (no change)
- Njxb (no change)

- Transcriptional regulation:
  - Cellular differentiation
  - Cell survival
  - Retrograde transport
  - Axonal outgrowth
  - Apoptosis

- Transcriptional regulation

FIGURE a4.8: Progesterone-mediated oocyte maturation.

- Progesterone
  - Pgr (no change)
  - Pik3r3: -35 ± 3% (p = 0.0382)

- Hspcb: -17 ± 4% (p = 0.0484)

- Translation of maternal mRNAs

- Metaphase II arrest
  - Cdc27: -33 ± 5% (p = 0.0065)
  - Mad2l1: -29 ± 6% (p = 0.0495)

- Meiosis I
- Meiosis II entry and S phase omission
FIGURE a4.9: Antigen processing and presentation.

FIGURE a4.10: Prostate cancer.
FIGURE a4.11: Inositol phosphate metabolism.

FIGURE a4.12: Melanogenesis.
FIGURE a4.13: GnRH signalling pathway.

Gonadotropin-Releasing hormone 1

Gnas
-40 ± 4% (p=0.0025)

Atf4
(no change)

Pikb1
-32 ± 5% (p=0.0273)

Pla2g1b
-31 ± 4% (p=0.0120)

Jun
+119 ± 27% (p=0.0007)

Gonadotropins gene expression and secretion

FIGURE a4.14: RNA degradation.

3'-5' decay
Cytoplasmic deadenylation

Cnot4
-25 ± 6% (p=0.0187)

Cnot6i
-42 ± 6% (p=0.0040)

5'-3' decay
Decapping complex

Ddx6
-32 ± 5% (p=0.0304)
FIGURE a4.15: Wnt signalling pathway.

Canonical pathway

```
Wnt4  -45 ± 4% (p=0.0025)
   ↓
Ctnnb1 -21 ± 5% (p=0.0404)
   ↓
Tgf3/7 (no change)
   ↓
Proteolysis
   ↓
Jun +119 ± 27% (p=0.0007)
```

Wnt/Ca²⁺ pathway

```
Wnt5 (no change)
   ↓
Ptkb1 -32 ± 5% (p=0.0273)
   ↓
Ca²⁺
   ↓
Nfat5 (no change)
   ↓
Transcriptional regulation
```

FIGURE a4.16: Focal adhesion.

```
Rapgef1 -32 ± 5% (p=0.0106)
   ↓
Ptk2 (no change)
   ↓
Pkr33 -35 ± 3% (p=0.0382)
```

```
Actn3 +21 ± 6% (p=0.0073)
   ↓
Ctnnb1 -21 ± 5% (p=0.0404)
   ↓
Akt (no change)
   ↓
Arhgap5 -29 ± 7% (p=0.0450)
```

ECM-receptor signalling

Cytokine-cytokine receptorsignalling

Regulation of actin cytoskeleton

Cell motility

Cell proliferation

```
Jun +119 ± 27% (p=0.0007)
```
FIGURE a4.17: NOD-like receptor signalling pathway.
APPENDIX 5 – KEGG pathways identified through GSEA as being differentially regulated between PA- and PA with metformin and sodium salicylate-treated cells.

FIGURE a5.1: Metabolism of xenobiotics by cytochrome P450.

- Cyp2b2 (Cyp2b12) -27 ± 5% (p=0.0080)
- Cyp2b3 +32 ± 12% (p=0.0487)
- Cyp2c11 -34 ± 4% (p=0.0414)
- Cyp2c12 (Cyp2c40) -45 ± 5% (p=0.0019)
- Cyp2e1 -36 ± 9% (p=0.0480)
- Cyp3a9 +22 ± 6% (p=0.0002)

Glutathione s-transferases
- Gsta3 +15 ± 5% (p=0.0392)
- Yc2 +19 ± 7% (p=0.0380)
- Gstm1 -29 ± 4% (p=0.0001)
- Gstm3 -68 ± 2% (p=3.3x10^-7)
- Gstt2 -13 ± 4% (p=0.0338)
- Gstpf1 +20 ± 5% (p=0.0057)
- Mgst2 -17 ± 5% (p=0.0262)

Metabolism of napthalene, 1-nitronapthalene, bromobenzene, benz(a)pyrene, 1,1-dichloroethylene, trichloroethylene and 1,2-dibromoethane

FIGURE a5.2: Drug metabolism – cytochrome P450.

- Ugt2b1 (Ugt2b17) -25 ± 7% (p=0.0193)
- Ugt2b10 (Ugt2b34) -29 ± 8% (p=0.0317)
- Ugt2b4 (Ugt2b36) -25 ± 8% (p=0.0463)
- Fmo5 -18 ± 6% (p=0.0495)

- Cyp2b1 (Cyp2b12) -27 ± 5% (p=0.0080)
- Cyp2b3 +32 ± 12% (p=0.0487)
- Cyp2c11 -34 ± 4% (p=0.0414)
- Cyp2c12 (Cyp2c40) -45 ± 5% (p=0.0019)
- Cyp2e1 -36 ± 9% (p=0.0480)
- Cyp3a9 +22 ± 6% (p=0.0002)

Glutathione s-transferases
- Gsta3 +15 ± 5% (p=0.0392)
- Yc2 +19 ± 7% (p=0.0380)
- Gstm1 -29 ± 4% (p=0.0001)
- Gstm3 -68 ± 2% (p=3.3x10^-7)
- Gstt2 -13 ± 4% (p=0.0338)
- Gstpf1 +20 ± 5% (p=0.0057)
- Mgst2 -17 ± 5% (p=0.0262)

Metabolism of tamoxifen, cyclophosphamide and ifosfamide, citalopram, codeine and morphine, methadone, lidocaine, felbamate, carbamazepine and oxycarbamazepine and valporic acid.
FIGURE a5.3: Complement and coagulation cascades.
FIGURE a5.4: Porphyrin and chlorophyll metabolism.
FIGURE a5.5: Biosynthesis of unsaturated fatty acids.

n-3  

16:0  18:0

\( \text{Elov6} \)

\(-19 \pm 6\% (p=0.0265)\)

18:0  18:0

18:3  18:2

\( \text{Fads2} \)

\(-25 \pm 6\% (p=0.0239)\)

18:4  18:3

22:6  22:5

\( \text{Acoa1} \)

\(+15 \pm 5\% (p=0.0356)\)

24:6  24:5

Fatty acyl CoA Species (C12-C22)

\( \text{Acot1 (Cte1)} \)

\(+60 \pm 12\% (p=0.0003)\)

\( \text{Acot4 (Pte1B)} \)

\(+29 \pm 3\% (p=0.0226)\)

\( \text{Acot2 (Mte1)} \)

\(+29 \pm 9\% (p=0.0149)\)

\( \text{Acot5} \)

\(+40 \pm 10\% (p=0.0034)\)

CoA + Free fatty acid
FIGURE a5.6: Retinol metabolism.

FIGURE a5.7: Drug metabolism – other enzymes.

Metabolism of azathioprine and 6-mercaptopurine, irinotecan, fluorouracil and isoniazid.
FIGURE a5.8: Arachidonic acid metabolism.

11-Dehydro-TXB2
15-OXOETE 5-OXOETE

\[ \text{Tbxas1} \quad +23 \pm 8\% \quad (p=0.0382) \]

\[ \text{Gpx1} \quad -37 \pm 4\% \quad (p=1.7 \times 10^{-5}) \]

19(S)-HETE

\[ \text{Cyp2e1} \quad -36 \pm 9\% \quad (p=0.0480) \]

20-HETE

15-Keto-PGF2α

\[ \text{Cbr3} \quad +57 \pm 15\% \quad (p=0.0033) \]

Arachidonate

\[ \text{Cyp2c12 (Cyp2c40)} \quad -45 \pm 5\% \quad (p=0.0019) \]

\[ \text{Cyp2c11} \quad -34 \pm 4\% \quad (p=0.0414) \]

\[ \text{Cyp2c11} \quad -27 \pm 5\% \quad (p=0.0080) \]

\[ \text{Cyp2c12 (Cyp2c40)} \quad -45 \pm 5\% \quad (p=0.0019) \]

\[ \text{Cyp2b3} \quad +32 \pm 12\% \quad (p=0.0487) \]

11,14,15-THETA

16(R)-HETE

Dihydroxyepoxycosadecenoic acid

11,12,15-THETA

Tetrahydrofurandiol

FIGURE a5.9: Glutathione metabolism.

\[ \text{NADPH} \quad \text{NADP}^+ \]

Glutathione disulfide (GSSG) Glutathione (GSH)

\[ \text{Gpx1} \quad -37 \pm 4\% \quad (p=1.7 \times 10^{-5}) \]

Gluathione s-transferases

\[ \text{Gsta3} \quad +15 \pm 5\% \quad (p=0.0392) \]

\[ \text{Gct2} \quad +19 \pm 7\% \quad (p=0.0380) \]

\[ \text{Gstm1} \quad -29 \pm 4\% \quad (p=0.0001) \]

\[ \text{Gstm3} \quad -68 \pm 2\% \quad (p=3.3 \times 10^{-7}) \]

\[ \text{Gstt2} \quad -13 \pm 4\% \quad (p=0.0338) \]

\[ \text{Gstp1} \quad +20 \pm 5\% \quad (p=0.0057) \]

\[ \text{Mgst3} \quad -17 \pm 5\% \quad (p=0.0262) \]

Acetyl-CoA

Marcapturic acid

R-S-Glutathione
FIGURE a5.10: Systemic lupus erythematosus.
FIGURE a5.11: Butanoate metabolism.

FIGURE a5.12: Synthesis and degradation of ketone bodies.
FIGURE a5.13: Fatty acid metabolism.

Fatty acid biosynthesis

Hexadecanoate (fatty acid)

Acadvl
-21 ± 4% (p=0.0078)

Ehhadh
+108 ± 24% (p=0.0004)

Ehhadh
+108 ± 24% (p=0.0004)

Hadhsdc
+9 ± 3% (p=0.0354)

Acaa1
+15 ± 5% (p=0.0356)

Acat2
-37 ± 4% (p=8.8x10^-8)

CoA

Ehhadh
+108 ± 24% (p=0.0004)

Hadhsdc
+9 ± 3% (p=0.0354)

Acetoacetyl-CoA

Synthesis and degradation of ketone bodies

Acaa1
+15 ± 5% (p=0.0356)

Acat2
-37 ± 4% (p=8.8x10^-8)

Acetyl-CoA

Citrate cycle
FIGURE a5.14: Sphingolipid metabolism.

- **Ppap2b**
  - $-36 \pm 6\% \ (p=0.0014)$
- **Acer1**
  - $-33 \pm 5\% \ (p=0.0398)$
- **Acer2**
  - $+36 \pm 10\% \ (p=0.0086)$
- **Glu**
  - $-16 \pm 4\% \ (p=0.0056)$

**Additional Notes**

- Similar to Mdes protein: $-34 \pm 8\% \ (p=0.0182)$
- Neutrophil protein: $-55 \pm 6\% \ (p=0.0009)$

**Diagram Components**

- **Sphingosine-1-P**
- **Sphingosine**
- **Ceramide**
- **Ceramide-P**
- **Sphingomyelin**
- **Diglactosylceramide**
- **Glc**
- **GM4**
- **Dihydroceramide**
- **Ppap2b**
- **Phosphoethanolamine**
- **Dihydrophosphoglycerine**
FIGURE a5.15: Linoleic acid metabolism.

FIGURE a5.16: Nicotinate and nicotinamide metabolism.
FIGURE a5.17: Valine, leucine and isoleucine degradation.

Leucine → Isoleucine → Valine

3-Hydroxy-isovaleryl-CoA

Hmgcs1
-23 ± 4% (p=0.0055)

Hmgcs2
-27 ± 6% (p=0.0193)

Acetyl-CoA

Acetoacetyl-CoA → Citrate cycle

Ehhadh
+108 ± 24% (p=0.0004)

Acoa1
+15 ± 5% (p=0.0356)

Acat1
-37 ± 4% (p=8.8x10^-6)

Acat2
-37 ± 4% (p=8.8x10^-6)

Hadhsc
+9 ± 3% (p=0.0354)
**FIGURE a5.18: RIG-1-like receptor signalling pathway.**

- **Viral RNAs**
  - **Ddx58**
    - $-22 \pm 6\% \ (p=0.0159)$
  - **Gip2**
    - $-65 \pm 3\% \ (p=2.0 \times 10^{-4})$
    - Binds to RIG-1 complex following IFNα or IFNβ stimulation

- **RIG-1 Complex**
  - **Ip51** (mitochondrial adaptor protein)
    - (no change)

- **Mapk11**
  - $+41 \pm 8\% \ (p=0.0002)$
  - Induces transcription of Inflammatory cytokines

- **Fadd**
  - $-23 \pm 4\% \ (p=0.0064)$
  - Caspase 8
    - (no change)

- **Irf7**
  - $-25 \pm 5\% \ (p=0.0061)$
  - Induces transcription of Type 1 interferons

- **Cxcl10**
  - $-57 \pm 3\% \ (p=0.0021)$
  - Induces transcription of Inflammatory cytokines

- **Nfkbia**
  - $-21 \pm 4\% \ (p=0.0083)$

- **Nfkb**
  - (no change)
FIGURE a5.19: Pentose and glucuronate interconversions.

FIGURE a5.20: Primary bile acid synthesis.
APPENDIX 6 – Side chain determination of PI 38:5 using the production ion scan application of liquid chromatography, electrospray ionisation-tandem mass spectrometry (LC ESI-MS/MS) analysis of intracellular lipid fractions.

FIGURE a6.1: Identification of the side chains of PI38:5 in human plasma as proof of principle. (a) Precursor ion scan for identification of PI38:5 by mass and retention time. (b) Product ions for identification of side chain fragments.
FIGURE a6.2: Identification of the side chains of PI38:5 in vehicle-treated cells. (a) Precursor ion scan for identification of PI38:5 by mass and retention time. (b) Product ions for identification of side chain fragments.
FIGURE a6.3: Identification of the side chains of PI38:5 in PA-treated cells.  
(a) Precursor ion scan for identification of PI38:5 by mass and retention time.  
(b) Product ions for identification of side chain fragments.
FIGURE a6.4: Identification of the side chains of PI38:5 in PAMN-treated cells. (a) Precursor ion scan for identification of PI38:5 by mass and retention time. (b) Product ions for identification of side chain fragments.
References


[21] Kristensen JS, Frandsen KB, Bayer T, Muller PG (2000) Compared with repaglinide sulfonylurea treatment in type 2 diabetes is associated with a 2.5-fold increase in symptomatic hypoglycemia with blood glucose levels < 45 mg/dl. Diabetes 49: A131-A131


cells by a mechanism involving protein kinase C and nuclear factor-kappaB activation. Endocrinology 147: 552-561


Turinsky J, O'Sullivan DM, Bayly BP (1990) 1,2-Diacylglycerol and ceramide levels in insulin-resistant tissues of the rat in vivo. J Biol Chem 265: 16880-16885


257


Meikle PJ, Christopher MJ (2011) Lipidomics is providing new insight into the metabolic syndrome and its sequelae. Curr Opin Lipidol


Mehnert H, Seitz W (1958) [Further results of diabetes therapy with blood sugar lowering biguanides]. Munch Med Wochenschr 100: 1849-1851


260


[177] Ikeda T (2011) Drug-induced idiosyncratic hepatotoxicity: prevention strategy developed after the troglitazone case. Drug Metab Pharmacokinet 26: 60-70


[179] Chen QM, Xie MZ (1986) [Studies on the hypoglycemic effect of Coptis chinensis and berberine]. Yao Xue Xue Bao 21: 401-406


Kotronen A, Velagapudi VR, Yetukuri L, et al. (2009) Serum saturated fatty acids containing triacylglycerols are better markers of insulin resistance than total serum triacylglycerol concentrations. Diabetologia 52: 684-690

Kamili A, Wat E, Chung RW, et al. (2010) Hepatic accumulation of intestinal cholesterol is decreased and fecal cholesterol excretion is increased in mice fed a high-fat diet supplemented with milk phospholipids. Nutr Metab (Lond) 7: 90


Holub BJ, Piekarski J (1979) The formation of phosphatidylinositol by acylation of 2-acyl-sn-glycero-3-phosphorylinositol in rat liver microsomes. Lipids 14: 529-532


