Liver Molecular Mechanisms Involved in Type 2 Diabetes:  
An Investigation Using Roux-en Y Gastric Bypass as a Human 
Model of Diabetes Remission 

By 
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Abstract

Type 2 diabetes mellitus is a chronic disease characterised by progressive insulin resistance and loss of β-cell function. An incomplete understanding of its pathogenesis is hindering the effective treatment of this disease. Roux-en Y gastric bypass surgery (RYGB); however, causes rapid remission of liver insulin resistance and type 2 diabetes, and therefore affords us an opportunity to examine some fundamental characteristics of these conditions. Gathering evidence suggests that liver insulin resistance may be a crucial contributor to development of diabetes. In this thesis, we used liver biopsies taken before, and in some individuals after, RYGB surgery to explore or identify several molecular processes involved in the pathogenesis of type 2 diabetes. The study cohort included individuals with normal glucose tolerance and others with type 2 diabetes.

Ecto-nucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) may cause insulin resistance through its inhibitory action on insulin signalling. This thesis provides evidence to the contrary, suggesting that liver ENPP1 is not a contributor to liver insulin resistance. We found liver ENPP1 protein abundance was lower in individuals with type 2 diabetes than in those with normal glucose tolerance, and increased after RYGB surgery in those individuals who had remission of diabetes. ENPP1 positively correlated with insulin sensitivity at the liver which is contrary to what others have reported in muscle and adipose tissue. We reasoned that our findings are likely due to the hypothesized role of ENPP1 as a natural modulator of insulin signalling and the unique role the liver has in insulin processing.

Changes in the expression ratio of insulin receptor (IR) isoform A (IR-A) and B (IR-B) have previously been implicated in the pathogenesis of type 2 diabetes. The metabolically active IR-B isoform has been shown to predominate in the liver, with the liver IR-B:A ratio being reported to be 9.8. By assaying levels of IR-A and IR-B mRNA expression we found that the ratio of liver IR-B:A was abnormally low in individuals with type 2 diabetes (5.2) and increased with remission of diabetes (5.4 to 8.6).
The change in ratio was due to a diminished IR-A expression following remission of diabetes. Further work with an *in vitro* cell model showed that insulin’s ability to inhibit gluconeogenesis in Hep G2 cells overexpressing IR-A was reduced, suggesting that the altered liver IR-B:A ratio observed in diabetes may have a detrimental effect on glucose homeostasis.

Access to liver tissue before and after RYGB surgery afforded a rare opportunity to observe changes in liver gene expression before and after remission of diabetes. Using gene microarray analysis, we showed that the majority of genes that were differentially regulated after RYGB surgery are involved in lipotoxicity, inflammation, and ER stress, particularly in those individuals who had remission of type 2 diabetes. Although there were many significant observations, the apparent inter-organ communication between the liver and the pancreas presents a hitherto unconfirmed relationship whereby the liver can not only mediate pancreatic β cell size, but can also regulate insulin secretion. This work has identified a mechanism which may be exploited to develop novel treatments for type 2 diabetes.

In conclusion this thesis describes several novel findings with respect to the pathogenesis of type 2 diabetes. It provides novel data on the role of ENPP1 and IR isoforms in insulin signalling which have furthered our understanding of the insulin signalling pathway. In addition, microarray gene analysis in liver tissue from before and after improvements in insulin resistance and remission of type 2 diabetes has allowed us to identify several candidate genes that are worthy of further investigation.
Acknowledgements

First I would like to thank Dr Mark Hayes and Professor Richard Stubbs for the support and guidance they have offered over the course of my study. They offered freedom rarely afforded to other PhD students which allowed me to develop into an independent and capable scientist. Dr Hayes’s assistance regarding experimental design and study direction was invaluable and critical to the completion of this work. Additional thanks are given to Professor Richard Stubbs, without whom none of the work presented here would be possible. His vision and dedication to the scientific method formed the extensive database and collection of human samples that was the fundamental basis for my thesis.

I would also like to acknowledge our research collaborators that have contributed significantly to the completion of my work. I would like to thank Professor C. Ronald Kahn and staff at the Joslin Diabetes Centre, Boston, MA, US for providing the IR-B isoform expression vector and Professor Conan Fee and the staff at the Biomolecular Interaction Centre for sequencing and validating said vector. Professor Eric Hoffman and the staff at the Research Centre for Genetic Medicine, Children’s Research Hospital, Washington DC, US for conducting the microarray gene analysis and Professor Parry Guilford at the Cancer Genetics Laboratory, Department of Biochemistry, University of Otago, Dunedin for allowing me to use their ABI Prism® 7900HT Sequence Detection System. I would also like to thank the many organisations that have provided funding, without which none of my work would be possible. They include; the Wakefield Biomedical Research Unit, the Wakefield Gastroenterology Research Trust and the Wellington Medical Research Foundation.

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Dissemination of Work

Publications

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Conference Proceedings and Presentations


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<th>Description</th>
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<tbody>
<tr>
<td>NGT</td>
<td>Individuals who had normal glucose tolerance (n=19)</td>
</tr>
<tr>
<td>IGT</td>
<td>Individuals who had impaired glucose tolerance (n=9)</td>
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<tr>
<td>T2DM</td>
<td>Individuals who had type 2 diabetes (n=27)</td>
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<td>sNGT</td>
<td>Individuals who had a second liver biopsy and normal glucose tolerance (n=8)</td>
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<tr>
<td>sT2DM</td>
<td>Individuals who had a second liver biopsy and type 2 diabetes (n=8)</td>
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**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A2M</td>
<td>Alpha-2-macroglobulin</td>
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<td>Long-chain-fatty-acid--CoA ligase 4</td>
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<td>CYP2C19</td>
<td>Cytochrome P450 2C19</td>
</tr>
<tr>
<td>DBH</td>
<td>Dopamine beta-hydroxylase</td>
</tr>
<tr>
<td>DDIT4</td>
<td>DNA-damage-inducible transcript 4</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DJB</td>
<td>Duodenal-jejunal bypass surgery</td>
</tr>
<tr>
<td>DM1</td>
<td>Myotonic Dystrophy type 1</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DUSP1</td>
<td>Dual specificity phosphatase 1</td>
</tr>
<tr>
<td>EEF1G</td>
<td>Eukaryotic translation elongation factor 1 gamma</td>
</tr>
<tr>
<td>ENO3</td>
<td>Enolase 3 (beta, muscle)</td>
</tr>
<tr>
<td>ENPP1</td>
<td>Ecto-nucleotide pyrophosphatase/phosphodiesterase</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal regulated kinase 1/2</td>
</tr>
<tr>
<td>FADS1</td>
<td>Fatty acid desaturase 1</td>
</tr>
<tr>
<td>FASN</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FIRKO</td>
<td>Fat insulin receptor knockout mice</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>FGL1</td>
<td>Fibrinogen-like protein 1</td>
</tr>
<tr>
<td>FNDC5</td>
<td>Fibronectin type III domain containing 5</td>
</tr>
<tr>
<td>FOSB</td>
<td>FBJ murine osteosarcoma viral oncogene homolog B</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>GADD45B</td>
<td>Growth arrest and DNA damage-inducible protein GADD45 beta</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>HbA1c</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>HBA2</td>
<td>Hemoglobin, alpha 2</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoprotein</td>
</tr>
<tr>
<td>H19</td>
<td>H19, imprinted maternally expressed transcript</td>
</tr>
<tr>
<td>HEPACAM</td>
<td>Hepatocyte cell adhesion molecule</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>Homeostatic model assessment-insulin resistance</td>
</tr>
<tr>
<td>HPRT1</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>HPS5</td>
<td>Hermansky-Pudlak syndrome 5 protein</td>
</tr>
<tr>
<td>HSD17B11</td>
<td>Estradiol 17-beta-dehydrogenase 11</td>
</tr>
<tr>
<td>IDE</td>
<td>Insulin degrading enzyme</td>
</tr>
<tr>
<td>IDF</td>
<td>International Diabetes Federation</td>
</tr>
<tr>
<td>IFI6</td>
<td>Interferon alpha-inducible protein 6</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>Insulin-like growth factor 1 receptor</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IGF-II</td>
<td>Insulin-like growth factor 2</td>
</tr>
<tr>
<td>IGFALS</td>
<td>Insulin-like growth factor-binding protein complex acid labile subunit</td>
</tr>
<tr>
<td>IGFBP2</td>
<td>Insulin-like growth factor-binding protein 2</td>
</tr>
<tr>
<td>IGFBP5</td>
<td>Insulin-like growth factor-binding protein 5</td>
</tr>
<tr>
<td>IGJ</td>
<td>Immunoglobulin J chain</td>
</tr>
<tr>
<td>IGLL1</td>
<td>Immunoglobulin lambda-like polypeptide 1</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
</tr>
<tr>
<td>IL32</td>
<td>Interleukin-32</td>
</tr>
<tr>
<td>IL1RN</td>
<td>Interleukin-1 receptor antagonist protein</td>
</tr>
<tr>
<td>IL1RAP</td>
<td>Interleukin-1 receptor accessory protein</td>
</tr>
<tr>
<td>iLIRKO</td>
<td>Inducible liver insulin receptor knockout mice</td>
</tr>
<tr>
<td>IRKO</td>
<td>Insulin receptor knockout mice</td>
</tr>
<tr>
<td>INHBE</td>
<td>Inhibin, beta E</td>
</tr>
<tr>
<td>IR</td>
<td>Insulin receptor</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IR-A</td>
<td>Insulin receptor isoform A</td>
</tr>
<tr>
<td>IR-B</td>
<td>Insulin receptor isoform B</td>
</tr>
<tr>
<td>IR-B:A</td>
<td>Insulin receptor isoform B to A ratio</td>
</tr>
<tr>
<td>JUN</td>
<td>Jun proto-oncogene</td>
</tr>
<tr>
<td>JUND</td>
<td>Transcription factor jun-D</td>
</tr>
<tr>
<td>KLF6</td>
<td>Kruppel-like factor 6</td>
</tr>
<tr>
<td>LAGB</td>
<td>Lap Band adjustable gastric banding</td>
</tr>
<tr>
<td>LEPR</td>
<td>Leptin receptor</td>
</tr>
<tr>
<td>LGALS4</td>
<td>Galectin-4</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
</tr>
<tr>
<td>LIRKO</td>
<td>Liver insulin receptor knockout mice</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LPA</td>
<td>Apolipoprotein(a)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MAP2K1</td>
<td>Dual specificity mitogen-activated protein kinase kinase 1</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MIRKO</td>
<td>Muscle insulin receptor knockout mice</td>
</tr>
<tr>
<td>MLLT10</td>
<td>Protein AF-10</td>
</tr>
<tr>
<td>MPT</td>
<td>Multivariate permutation test</td>
</tr>
<tr>
<td>MTE</td>
<td>Metallothionein</td>
</tr>
<tr>
<td>MT1A</td>
<td>Metallothionein-1A</td>
</tr>
<tr>
<td>MT1F</td>
<td>Metallothionein 1F</td>
</tr>
<tr>
<td>MT1G</td>
<td>Metallothionein-1G</td>
</tr>
<tr>
<td>MT1H</td>
<td>Metallothionein 1H</td>
</tr>
<tr>
<td>MT1M</td>
<td>Metallothionein 1M</td>
</tr>
<tr>
<td>MT2A</td>
<td>Metallothionein-2</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NIRKO</td>
<td>Neuron insulin receptor knockout mice</td>
</tr>
<tr>
<td>NR4A2</td>
<td>Nuclear receptor subfamily 4 group A member 2</td>
</tr>
<tr>
<td>OSTalpha</td>
<td>OSTalpha protein</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCK1</td>
<td>Phosphoenolpyruvate carboxykinase 1 (gene)</td>
</tr>
<tr>
<td>PEI</td>
<td>Polyethyleneimine</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase 1 (protein)</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PLIN2</td>
<td>Perilipin-2</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene difluoride</td>
</tr>
<tr>
<td>P4HA1</td>
<td>Prolyl 4-hydroxylase subunit alpha-1</td>
</tr>
<tr>
<td>PYROXD2</td>
<td>Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2</td>
</tr>
<tr>
<td>RCAN1</td>
<td>Calcipressin-1</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>REST</td>
<td>Relative expression software tool</td>
</tr>
<tr>
<td>RFTN1</td>
<td>RFTN1 protein</td>
</tr>
<tr>
<td>RHOB</td>
<td>Rho-related GTP-binding protein RhoB</td>
</tr>
<tr>
<td>RND3</td>
<td>Rho-related GTP-binding protein RhoE</td>
</tr>
<tr>
<td>RPL10A</td>
<td>60S ribosomal protein L10</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Reverse transcription quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>RVM</td>
<td>Random variance model</td>
</tr>
<tr>
<td>RYGB</td>
<td>Roux-en Y gastric bypass</td>
</tr>
<tr>
<td>SAA1</td>
<td>Serum amyloid A-1 protein</td>
</tr>
<tr>
<td>SDS</td>
<td>Serine dehydratase</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>Serpine peptidase inhibitor, clade E, member 1</td>
</tr>
<tr>
<td>SERPINA11</td>
<td>Serpin A11</td>
</tr>
<tr>
<td>SGK1</td>
<td>Serine/threonine-protein kinase</td>
</tr>
<tr>
<td>SHBG</td>
<td>Sex hormone-binding globulin</td>
</tr>
<tr>
<td>SIK1</td>
<td>Serine/threonine-protein kinase</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SLC2A3</td>
<td>Solute carrier family 2, facilitated glucose transporter member 3</td>
</tr>
<tr>
<td>SLC25A25</td>
<td>Calcium-binding mitochondrial carrier protein SCaMC-2</td>
</tr>
<tr>
<td>SLC29A4</td>
<td>Equilibrative nucleoside transporter 4</td>
</tr>
<tr>
<td>SLC3A1</td>
<td>Neutral and basic amino acid transport protein rBAT</td>
</tr>
<tr>
<td>SLC22A10</td>
<td>Solute carrier family 22 member 10</td>
</tr>
<tr>
<td>SNAI2</td>
<td>Zinc finger protein SNAI2</td>
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<tr>
<td>SPTBN1</td>
<td>Spectrin beta chain, non-erythrocytic 1</td>
</tr>
<tr>
<td>SQLE</td>
<td>Squalene monooxygenase</td>
</tr>
<tr>
<td>SRD5A2</td>
<td>3-oxo-5-alpha-steroid 4-dehydrogenase 2</td>
</tr>
<tr>
<td>SYT7</td>
<td>Synaptotagmin-7</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethynediame</td>
</tr>
<tr>
<td>TLDL</td>
<td>Taqman low density array</td>
</tr>
<tr>
<td>TMEM45B</td>
<td>Transmembrane protein 45B</td>
</tr>
<tr>
<td>TMEM154</td>
<td>Transmembrane protein 154</td>
</tr>
<tr>
<td>TRIB1</td>
<td>Tribbles homolog 1</td>
</tr>
<tr>
<td>TP53INP1</td>
<td>Tumor protein p53 inducible nuclear protein 1</td>
</tr>
<tr>
<td>UPP2</td>
<td>Uridine phosphorylase 2</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VBG</td>
<td>Vertical banded gastroplasty</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
</tr>
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</table>
CHAPTER ONE: The pathology of type 2 diabetes
1.1 Type 2 diabetes mellitus: a disease for modern man

The need to feed is a fundamental driving force of life. Humans need a steady intake of nutrients to provide the fuel needed to keep our central nervous system performing. Because of the irregular availability of nutrients we have developed a mechanism which promotes anabolism when nutrient supply exceeds demand or catabolism when nutrient intake can not meet demand. Insulin secretion and action are integral parts of this mechanism. Secreted by the pancreatic \( \beta \)-cells in response to nutrient intake, insulin promotes carbohydrate uptake and storage for immediate use and change of carbohydrate and protein to lipids for long term storage. For millennia this system has served development of \textit{Homo sapiens} well. But recently this powerful survival mechanism has turned on humanity. Because of our ability to alter our environment in shorter time frames, we have gone from having low nutrient availability and high physical demand to abundant nutrient supply and relatively little physical demand.

Obesity and type 2 diabetes mellitus are now endemic and the world is grappling with an imminent crisis. The global prevalence of type 2 diabetes is predicted to increase from \( \sim 285 \) million in 2010 to \( \sim 552 \) million by the year 2030.\textsuperscript{1, 2} A chronic and progressive disease, type 2 diabetes is a manifestation of a much broader underlying disorder called Metabolic Syndrome (MetS). The International Diabetes Federation (IDF) has defined MetS as consisting of abdominal obesity, impaired glucose tolerance, hyperinsulinaemia, dyslipidaemia and hypertension.\textsuperscript{3} Although there have been various definitions of MetS due to its association with both diabetes and cardiovascular disease, a recent joint consensus statement has recommended guidelines for clinical diagnosis.\textsuperscript{4} Nonetheless, the IDF classification of metabolic syndrome is more predictive for development of impaired fasting glucose than classifications by other organisations like the National Cholesterol Education Program-Third Adult Treatment Panel (NCEP-ATPIII).\textsuperscript{5}

The goal of conventional treatment for type 2 diabetes is strict glycaemic control that is meant to prevent and/or reduce the risk of progression to end stage organ disease such as myocardial infarctions, stroke, retinopathy and nephropathy.\textsuperscript{6}
Even so, studies have shown that in the United States only 50% of individuals with type 2 diabetes reach glycaemic control targets suggesting many go on to develop end stage organ disease. Additionally, of the three large scale clinical trials (VADT, ACCORD and ADVANCE) that used intensive therapy to lower glycated hemoglobin levels (HbA1c), only one showed a 10% reduction of cardiovascular events. The burden this will place on already struggling national healthcare systems is enormous. In New Zealand, expenditure on treating type 2 diabetes is expected to increase from 600 million in 2007 to 1.3 billion by 2016.

A drastic shift in the way we approach treatment of type 2 diabetes and its complications is needed. Rather than controlling the symptoms, treatments that address the underlying pathogenesis must be developed. Unfortunately, despite decades of intensive research into diabetes the exact mechanisms and precise sequence of events behind its pathogenesis are still unclear. Although extensive use of animal models and in vitro cell culture has elucidated the large majority of the pathways involved in insulin signalling, the molecular lesions that cause the characteristic insulin resistance that contributes to the development of type 2 diabetes are still unknown.

However, the scientific community is becoming increasingly aware of what bariatric surgeons have known since 1995. That bariatric surgery can cause remission of type 2 diabetes in 50-95% of individuals depending on procedure type. Bariatric surgery is so effective that in 2011 the IDF released a position statement recommending bariatric surgery as a treatment for type 2 diabetes in morbidly obese individuals.

The work presented here is centred around the effects bariatric surgery has on type 2 diabetes. Specifically, gastric bypass surgery was used as a human model to provide novel insights on molecular processes previously implicated in the pathogenesis of type 2 diabetes and to search for as yet unknown mechanisms that may contribute to this disease. The following literature review justifies the work done and it begins with a description of the two main abnormalities present in type 2 diabetes, namely insulin resistance and impaired insulin secretion.
1.2 Insulin resistance and impaired insulin secretion

Type 2 diabetes is thought to be the culmination of progressive insulin resistance and loss of pancreatic β-cell function. In normal individuals a square wave increase in plasma glucose concentration causes insulin to be released from the pancreas which acts to increase glucose uptake and storage while decreasing endogenous glucose production. Insulin resistance describes the impaired biological response to insulin action and is characterized by progressively higher insulin concentrations, which is a hallmark feature of type 2 diabetes. 16, 17

Insulin resistance is one of the earliest detectable pathologies in individuals who progress from normal glucose tolerance to impaired glucose tolerance and finally to type 2 diabetes. 18 It contributes to the characteristic fasting and postprandial hyperglycaemia seen in type 2 diabetes by causing dysregulated hepatic glucose production and impaired glucose uptake respectively. 19, 20 However, even though insulin resistance can be present 10-20 years prior to diabetes, 18 fasting hyperglycaemia does not manifest until there are defects in insulin secretion (Figure 1.2-1). 20-23

![Figure 1.2-1](image_url). Relationship between pancreatic beta cell function and insulin sensitivity. As the beta cells fail to compensate for the decreased insulin sensitivity by increasing insulin secretion individuals will develop impaired glucose tolerance and eventually type 2 diabetes (adapted from Kahn et al. 1993) 24. IGT; Impaired glucose tolerance, NGT; Normal glucose tolerance, T2DM; type 2 diabetes mellitus.
Impaired insulin secretion is caused by abrogation of the frequency and amplitude of insulin release in response to glucose. It has been observed in both individuals with normal fasting plasma glucose and individuals with impaired glucose tolerance suggesting it can be present well before development of overt hyperglycaemia.\textsuperscript{25} Consequently, failure of the $\beta$-cell to compensate for the insulin resistance is thought to be a pre-requisite step for development of impaired glucose tolerance and type 2 diabetes.\textsuperscript{26-28}

Insulin resistance is often used solely to describe the inability of insulin to regulate glucose homeostasis. However, the actions of insulin are broad and require consideration in order to fully appreciate the importance of insulin resistance and its role in type 2 diabetes. The following sections will detail insulin action from secretion through to binding of the insulin receptor and transmission of the signal. It will outline the rationale of why the liver may have overriding importance in type 2 diabetes and the possible mechanisms behind insulin resistance.

### 1.3 Insulin action: from secretion to binding of the insulin receptor

The insulin signalling system can be divided into three sequential components; 1) insulin production and secretion, 2) hepatic insulin clearance and 3) insulin binding of the insulin receptor and signal transmission.

#### 1.3.1 Insulin secretion

Insulin is produced in the $\beta$-cells of the pancreatic islets of Langerhans as a 5808 Da polypeptide hormone. It is first synthesized as a single polypeptide preproinsulin and shortly after is processed in the endoplasmic reticulum to proinsulin before it is sent to the trans-Golgi network.\textsuperscript{25} Proinsulin consists of an A and B chain linked together by disulfide bonds and a C-peptide bridge. Endopeptidases cleave off the C-peptide bridge creating mature insulin which is packed into granules and stored on the plasma membrane. Metabolic signals result in exocytosis of the granules and release of insulin into the circulation. The main mechanism for eliciting insulin secretion is the
phosphorylation of glucokinase, which is mediated by glucose upon its entry into the β-cells through the GLUT 2 channel.25

There are two distinct mechanisms involved in insulin secretion from the pancreas into the hepatic portal vein. Firstly, insulin secretion occurs in discrete secretory pulses every four minutes.29, 30 The oscillatory nature of insulin secretion has been shown to be important in insulin action.31-33 Consequently, a decreased pulse mass is thought to be a contributing factor to the impaired insulin secretion seen in type 2 diabetes.34-36

Insulin secretion is also biphasic in response to a glucose stimulus (Figure 1.3-1).37-39 The initial spike in glucose-induced insulin release is referred to as the rapid or first-phase. It is characterized by a rapid increase in insulin secretion (or insulin pulse mass) which can last for up to 10 minutes.40 Continued exposure of the β-cell to glucose leads to a slower but sustained second-phase of insulin secretion that occurs within 10-20 minutes after glucose exposure and can last for several hours.40 It is thought that insulin-containing granules exist as two functionally distinct pools that are distinguished by their proximity to the plasma membrane.41 The pool closest to the plasma membrane (<5% of mature insulin-containing granules) is thought to be responsible for the glucose-induced first-phase insulin release,41, 42 whereas the sustained second-phase is a consequence of stored insulin-containing granules and de novo insulin synthesis.41, 43, 44

First-phase insulin secretion is important in maintaining normoglycaemia and is thought to be central to the metabolic shift from a fasting to a postprandial state.40 Studies have shown that it is required for the efficient suppression of hepatic glucose production45-47 and may also prime peripheral insulin sensitive-tissues to increase glucose uptake.39 An abrogated first-phase response has been shown to be associated with impaired glucose tolerance48-51 and may be an early event in the development of type 2 diabetes.40, 52, 53
Figure 1.3-1. Schematic diagram of biphasic insulin secretion after square wave increase in plasma glucose concentration. Insulin secretion occurs in discrete secretory pulses and is oscillatory in nature. A square wave increase in glucose concentration causes a rapid increase in insulin secretion (first phase) followed by slower but sustained secretion (second phase) (adapted from Pratley and Weyer, 2001).40

Furthermore the suppression of hepatic glucose production is thought to be primarily due to a decrease in gluconeogenesis,54 which suggests that the first-phase insulin secretion has more of an effect on the liver as opposed to peripheral tissue. Considering hepatic gluconeogenesis is a characteristic pathology in type 2 diabetes23 the importance of the liver as a key organ in regulating insulin action and glucose homeostasis should not be overlooked.

1.3.2 Hepatic insulin clearance

Once released from the pancreas, insulin travels in pulses via the hepatic portal vein to the liver where it binds to the insulin receptor and is either degraded or released into the systemic circulation. The liver is the only organ which is exposed to insulin concentrations that can range in amplitude between 1,000-5,000 pmol/l.55 The level of insulin in the systemic circulation is only ~1% of that presented at the liver.55 Although the lower insulin concentration in the systemic circulation is due in part to the five fold dilution of hepatic portal vein blood by the systemic circulation, the liver has also been shown to have a key role in extracting insulin from the circulation.56
Insulin clearance at the liver is crucial to maintaining an appropriate systemic insulin concentration. Accordingly, the liver is thought to respond minute by minute to changes in insulin concentration by decreasing hepatic insulin clearance in response to increased demand for insulin and vice versa. Hepatic insulin clearance has been found to be diminished in obesity and type 2 diabetes. Consequently, it has been suggested that diminished insulin clearance may be a potential compensatory mechanism that maintains the high systemic insulin levels needed to maintain normoglycaemia in insulin resistant states.

1.3.3 Molecules involved in hepatic insulin clearance

Insulin clearance by the liver is a complex process that is a function of insulin internalization and degradation. The primary mechanism for hepatic insulin uptake is an insulin receptor-mediated process. However, insulin that is removed from the circulation is not necessarily degraded, with a significant amount of receptor bound insulin being released back into the circulation. Generally, 80% of total insulin in the body is bound to the insulin receptors on the liver, which extracts ~50% of the secreted insulin during first pass transit. In the last 15 years, major strides have been made in uncovering some of the molecules involved in insulin internalization and degradation. Although some of the functional characteristic of carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) and insulin degrading enzyme (IDE) have been identified, their involvement in the pathological process of type 2 diabetes remains to be profiled.

CEACAM-1 is involved in mediating the internalization of the insulin-insulin receptor complex. In addition to its role in insulin internalization and cell-cell adhesion it has also been found to suppress tumour growth in vivo and to downregulate the mitogenic effects of insulin. Insulin binding to the insulin receptor causes the IR tyrosine kinase to phosphorylate CEACAM-1. Surprisingly this action is specific to IR as its close relative the insulin like growth factor-1 receptor does not illicit a similar response. Specifically it is the C terminus of the β subunit of the insulin receptor that is required for insulin stimulated phosphorylation of CEACAM-1.
Liver-specific overexpression of a dominant negative (S503A) mutant of CEACAM-1, which is phosphorylation-deficient, impairs insulin clearance causing hyperinsulinaemia. This leads to secondary insulin resistance, abnormal glucose tolerance, increased fatty acids and visceral adiposity.\textsuperscript{65, 70} Likewise a CEACAM-1 null mutation (\textit{Cc}-\textsuperscript{-}\textit{f}) in mice causes impaired insulin clearance resulting in hyperinsulinaemia, hepatic steatosis and visceral adiposity.\textsuperscript{64} Evidently, CEACAM-1 has an important role in insulin action via regulation of its internalization and consequent hepatic clearance.

Hepatic insulin clearance is also dependent on IDE-mediated insulin degradation. Insulin degradation is a regulated process that ultimately inactivates and removes insulin from the circulation. Inhibitors of IDE have previously been proposed as a potential anti-diabetic therapy.\textsuperscript{71} In animal models of diabetes (Goto-Kakazaki rats) IDE was shown to be involved in the diabetic phenotype by contributing to insulin resistance and hyperglycaemia through an impaired ability to degrade insulin.\textsuperscript{72} Accordingly, mice lacking IDE developed hyperinsulinaemia leading to progressive insulin resistance and ultimately glucose intolerance,\textsuperscript{73} thus reflecting the progressive nature of insulin resistance and type 2 diabetes. Consequently, in a prospective case-cohort study, two IDE polymorphisms were associated with type 2 diabetes risk and were found to exert influence on insulin degradation, secretion and sensitivity.\textsuperscript{74} Like CEACAM-1, IDE is a major component of hepatic insulin clearance, which has previously been implicated in the pathogenesis of type 2 diabetes.

\textbf{1.3.4 Classical insulin target tissues and consequence of signal transmission}

Insulin exerts its metabolic effects by binding to the insulin receptor. As a multipotent hormone, insulin regulates several major biological processes which include glucose metabolism, lipid metabolism and protein turnover. In humans, maintaining a constant supply of glucose is crucial for continued biological functions due to the reliance of the brain on glucose as its major fuel source. Organs like the brain and cells like erythrocytes consume glucose evenly in fed and fasted states.\textsuperscript{75} Consequently, maintaining normoglycaemia in the face of variable availability of glucose is achieved by regulating glucose uptake and endogenous glucose production (Figure 1.3-2).
The three classic target tissues of insulin action are liver, muscle and fat (Figure 1.3-2). In fasting conditions the liver releases glucose via glycogenolysis and gluconeogenesis. In the postprandial state insulin levels rise which inhibits breakdown of glycogen via inhibition of glycogenolysis in muscle and liver tissue in addition to inhibiting gluconeogenesis in the liver. At the same time insulin stimulates glucose uptake by muscle and fat tissue. In addition to stimulating glucose uptake, insulin increases glucose storage as either glycogen or lipids in various tissues. The liver stores both glycogen and lipids, whereas muscle and adipose tissue store glycogen and lipid respectively.
Insulin also has indirect effects on glucose metabolism by decreasing the release of gluconeogenic precursors via regulation of lipid metabolism and protein turnover. Insulin inhibition of lipolysis at adipose tissue reduces the release of free fatty acids (FFA) and glycerol which contributes to the inhibition of hepatic glucose production. Likewise, insulin inhibition of proteolysis at muscle tissue decreases the release of amino acids necessary for gluconeogenesis.\textsuperscript{79,80}

1.3.5 Non-classical insulin target tissue

Although the classical insulin target tissues are the major regulators of insulin action, other tissue such as the central nervous system, vascular cells and pancreatic β-cells have also been found to have roles in insulin signalling.\textsuperscript{76} Insulin signalling in the brain is thought to be involved in appetite suppression,\textsuperscript{81,82} and has been shown to be abrogated in obese, insulin-resistant, normal glucose tolerant individuals.\textsuperscript{83} In vascular cells it is thought to stimulate vasodilatation and capillary recruitment thus enhancing glucose delivery and increasing muscle glucose uptake.\textsuperscript{84} In the β-cell insulin has been shown to activate transcription of both the insulin and glucokinase genes.\textsuperscript{85}

1.4 Insulin resistance in the classical insulin target tissues

Insulin resistance is defined as a reduced biological effect for any given concentration of insulin.\textsuperscript{86} The impact of insulin resistance extends far beyond its effect on glucose metabolism and is one of the fundamental physiological changes that lead to the cluster of abnormalities (type 2 diabetes, obesity, hypertension, dyslipidaemia, cardiovascular disease) seen in metabolic syndrome X.\textsuperscript{17} The measurement of insulin resistance in humans is a key component of managing metabolic syndrome and type 2 diabetes and is often approximated by methodologies that measure the biological action of insulin (insulin sensitivity). Insulin sensitivity is a quantitative measure of a specific action of insulin and is the reciprocal of insulin resistance.\textsuperscript{87}
1.4.1 Measurement of insulin resistance

Although the different methodologies employed for measuring insulin resistance are numerous and complex, they can be divided into two major groups.86 What follows is a brief description of two most common techniques used.

Non-dynamic methodologies take measurements during the steady state and can be based on glucose and insulin measurements alone. One such measurement is the Homeostasis model assessment (HOMA), which takes measurements during fasting conditions.88, 89 HOMA is a mathematical model that generates values for insulin sensitivity from fasting plasma glucose and insulin concentrations.88 As HOMA is based on the assumption of a feedback loop between the liver and the pancreas and is used in the fasting state, where normoglycaemia is regulated by hepatic glucose output, it is more indicative of hepatic insulin resistance. HOMA-IR values less than or equal to 2.4 have been shown to be representative of an insulin sensitive, normal glucose tolerant population.89-91

Dynamic measurements of insulin sensitivity are based on artificial disruption of the steady state and evaluation of the return to the steady state. An example technique is the euglycemic clamp technique.86 With this technique glucose levels are maintained constant with a variable intravenous infusion and insulin levels are elevated using a constant intravenous insulin infusion. Once the glucose levels are ‘clamped’ at a predetermined concentration the insulin resistance is inversely related to the glucose infusion rate necessary to maintain the required glucose concentration.92 Although the euglycaemic clamp is the gold standard for measuring insulin sensitivity in vivo, its execution is time consuming, and labour intensive.86 Consequently, because of its simplicity, HOMA is often used to estimate insulin resistance. Estimates of insulin resistance from HOMA correlated highly with estimates of insulin resistance using the euglycaemic clamp in two different studies (R_s =0.88, p<0.000188, R_s =0.85, p<0.0001)93 suggesting HOMA is a robust and appropriate method for estimating changes in insulin resistance.
1.4.2 Insulin resistance in peripheral tissue

Insulin resistance in the periphery can affect glucose disposal and lipolysis. Muscle insulin resistance is present when insulin-mediated glucose disposal through the GLUT4 channel is reduced to the lowest quartile of control subjects. Muscle tissue is responsible for 90% of glucose disposal in peripheral tissue. Because the euglycaemic clamp technique uses the glucose disposal rate to estimate insulin resistance, muscle tissue has been considered to be central to the development of type 2 diabetes. The progressive hyperinsulinaemia required to overcome the insulin resistance at the muscle is also thought to play a role in the development of metabolic syndrome. Furthermore, insulin resistance at adipose tissue leads to unregulated lipolysis and an increase in circulating FFAs, which promotes hepatic glucose output. This has lead some to suggest that the inability of insulin to inhibit lipolysis may be a major contributor to the increased hepatic glucose output seen in diabetes.

1.4.3 Insulin resistance at the liver

Of the three classical insulin target tissues, the liver is the primary organ that directly regulates metabolic homeostasis. Not only does it control insulin levels in the periphery via hepatic insulin clearance, but it is also the major organ that regulates normoglycaemia during fasting conditions and has a major role in lipid homeostasis. Uncontrolled hepatic gluconeogenesis has been shown to contribute to the fasting and overnight hyperglycaemia seen in individuals with type 2 diabetes.

Following an overnight fast, the liver of normal individuals produces glucose at an approximate rate of 2mg/kg per minute, whereas the liver of an individual with type 2 diabetes produces glucose at a rate of 2.5mg/kg per minute. As the diabetic state worsens there is a correlated increase between hepatic glucose production and fasting plasma glucose concentrations, despite the progressive (2.5-3 fold) increase in insulin concentration. Consequently, hepatic insulin resistance has been shown to adversely affect the direct suppressive effect of insulin on hepatic glucose production in type 2
diabetes. An abrogated first phase insulin response is also likely involved as it has been associated with impaired suppression of hepatic glucose production. Insulin resistance at the liver has multiple effects, of which, the ability to regulate lipid homeostasis has become increasingly relevant with regards to its role in metabolic syndrome. The dyslipidaemia that is often present in individuals with type 2 diabetes is characterized by hypertriglyceridaemia, raised LDL-cholesterol and a low HDL cholesterol profile. The overstimulation of lipogenesis at the liver due to hyperinsulinaemic conditions is thought to be a critical component of the overproduction of VLDL particles seen in type 2 diabetes. Consequently, the abnormal fat storage and ectopic fat deposition in other insulin target tissues has been suggested to have a role in the progressive nature of insulin resistance.

All three of the classical insulin target tissues have critical roles in regulating glucose homeostasis (Figure 1-4.1). However, the question of which insulin signalling tissues (if any) have overriding importance in the progression to type 2 diabetes is yet to be answered. Although in vivo studies in humans are useful, they are ultimately limited. The use of animal models allows researchers to target specific tissue and observe the sequential molecular changes that occur as one passes from normal glucose tolerance to type 2 diabetes.

Figure 1.4-1. A brief depiction of the key organs responsible for the unregulated hyperglycaemia seen in type 2 diabetes. A combination of impaired insulin secretion and insulin resistance causes increased glucose output in the liver, increased lipolysis in fat tissue and decreased glucose uptake in muscle tissue. (Adapted from DeFronzo, 2009)
1.5 Insulin target tissue relevance in regulating glucose homeostasis

Transgenic animal models have focused our understanding of the relevance of insulin target tissues in glucose homeostasis. Although muscle and fat tissue still have a fundamental role in the progression to type 2 diabetes, compelling data have highlighted the importance of the liver, pancreatic β-cell and brain.111

1.5.1 Transgenic animal models of tissue specific insulin resistance

Studies by Kahn and colleagues have been useful in determining the importance of specific insulin target tissue (Table 1-1).112-116 In these studies, tissue specific total insulin resistance was created by ablating the insulin receptor using Cre-loxP technology. Cre-loxP uses a bacterial recombinase (Cre) to excise a portion of DNA that has been tagged by two recognition sequences (lox).117 In the case of the insulin receptor gene, transgenic mice with lox sequences inserted either side of exon 4 of the insulin receptor are bred with transgenic mice expressing Cre recombinase. Cre recombinase expression is driven by a tissue specific promoter (e.g. albumin promoter in liver tissue),116 resulting in offspring that have a missense hepatic insulin receptor mRNA that codes for a non-signalling insulin receptor fragment.

Because of the importance of muscle glucose uptake in glucose homeostasis it was one of the first tissues to be investigated. Surprisingly, muscle insulin receptor knockout mice (MIRKO)114 did not have aberrant glucose homeostasis despite elevated plasma triglycerides and FFA. The inability of MIRKO muscle tissue to take up glucose was compensated by adipose tissue, which resulted in increased adiposity and weight gain.

Similarly, fat insulin receptor knockout mice (FIRKO)112 also had an unexpected phenotype. Not only did FIRKO mice have normal glucose homeostasis in addition to having improved insulin sensitivity; they were also protected against obesity-induced glucose intolerance and had increased longevity.
Table 1-1. Transgenic tissue specific mouse models of insulin resistance.

<table>
<thead>
<tr>
<th>Insulin Receptor knockout</th>
<th>Insulin Secretion</th>
<th>Insulin action</th>
<th>Glucose Homeostasis</th>
<th>Lipid homeostasis</th>
<th>Obesity</th>
<th>Glucose tolerance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muscle (MIRKO)(^{114})</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>↑FFA ↑Triglycerides</td>
<td>Obese</td>
<td>Normal</td>
</tr>
<tr>
<td>Fat (FIRKO)(^{112})</td>
<td>Normal</td>
<td>Improved insulin sensitivity</td>
<td>Normal↑</td>
<td>Normal↑↓Triglycerides</td>
<td>Lean*</td>
<td>Improved</td>
</tr>
<tr>
<td>Liver (LIRKO)(^{116})</td>
<td>Increased</td>
<td>Markedly insulin resistant</td>
<td>Hyperglycaemia</td>
<td>↓FFA ↓Triglycerides</td>
<td>-</td>
<td>Severely Impaired</td>
</tr>
<tr>
<td>B-cell (βIRKO)(^{115})</td>
<td>Impaired</td>
<td>Mildly insulin resistant</td>
<td>Normal</td>
<td>-</td>
<td>-</td>
<td>Impaired</td>
</tr>
<tr>
<td>Neuronal (NIRKO)(^{111})</td>
<td>Increased</td>
<td>Mildly insulin resistant</td>
<td>Normal</td>
<td>↑Triglycerides</td>
<td>Obese</td>
<td>Normal</td>
</tr>
</tbody>
</table>

* Protected against diet-induced obesity.

Liver insulin receptor knockout mice (LIRKO)\(^{116}\) showed severe glucose intolerance and insulin resistance. They had mild fasting hyperglycaemia and marked postprandial hyperglycaemia, which was thought to stem from the inability of insulin to inhibit hepatic glucose production. LIRKO mice also exhibited increased insulin secretion and impaired hepatic insulin clearance (due to loss of receptor-mediated insulin internalization and degradation) resulting in marked hyperinsulinaemia. Although this caused secondary whole body insulin resistance, as the mice aged the impaired glucose tolerance resolved. This suggested even though the liver appears to have more relevance than muscle and fat in insulin resistance, failure of pancreatic β-cell function is still necessary for progression to overt type 2 diabetes.

Pancreatic β-cell insulin receptor knockout (βIRKO)\(^{115}\) mice are characterized by one of the earliest defects found in individuals with type 2 diabetes, namely loss of glucose stimulated first phase secretion. As with humans, loss of the first-phase insulin response in βIRKO mice leads to impaired glucose tolerance that worsens with age. This was later shown to be caused by the inability of insulin to increase glucokinase gene transcription in the pancreas.\(^{85}\) The data from the βIRKO mouse shows that insulin resistance at the β-cell may be as critical as hepatic insulin resistance appears to be in the development of type 2 diabetes.
The brain has also been shown to have a critical role in regulating glucose homeostasis. Neuronal insulin receptor knockout mice (NIRKO)\textsuperscript{113} have whole body insulin resistance, hyperphagia and obesity. Likewise, decreasing insulin receptors in the rat hypothalamus not only induced hyperphagia and insulin resistance but also negatively affected the ability of insulin to regulate hepatic glucose output.\textsuperscript{118}

1.5.2 Interplay between key insulin target tissues in progression to type 2 diabetes: does the liver have overriding importance?

The transgenic animal models of Kahn and colleagues give great insight into tissue specific regulation of glucose homeostasis. One major conclusion from these animal models is that although some tissues (liver and pancreas) appeared more relevant in the pathogenesis of type 2 diabetes than others (muscle and fat), insulin resistance at a single tissue is not enough to generate diabetes. Consequently, several animal models have been developed that have defects in multiple tissues.

An animal model with targeted insulin resistance in both muscle and adipose tissue showed impaired glucose tolerance and impaired insulin secretion, but did not develop diabetes.\textsuperscript{119} Compounding β-cell insulin resistance with muscle insulin resistance (βIRKO-MIRKO) improved rather than worsened glucose tolerance.\textsuperscript{120} These data suggest that peripheral insulin resistance, even with impaired insulin secretion, is not enough to produce diabetes.

Simultaneous knockout of insulin receptors in liver and pancreatic β-cells; however, generated markedly diabetic mice providing further evidence of the importance of these tissues in type 2 diabetes.\textsuperscript{111} By the same token, animals that have whole body knockout of the insulin receptor (IRKO) can be rescued from death by reconstituting insulin receptor in certain tissues. Shortly after birth, IRKO mice develop severe hyperinsulinaemia followed by β-cell failure, diabetic ketoacidosis and death.\textsuperscript{121} Reconstituting insulin receptor in the liver, pancreatic β-cells, and brain rescues IRKO mice from neonatal death and prevents development of diabetes.\textsuperscript{122} Given these points, it is becoming increasingly apparent that insulin resistance at these three tissues is necessary for the development of type 2 diabetes.
Collectively, the data from the LIRKO, βIRKO and other animal models points to a tantalizingly unified theory in which insulin resistance at the liver causes whole body insulin resistance and ultimately type 2 diabetes. To test this hypothesis Escribano et al. (2009) created an inducible liver specific insulin receptor knockout mice model (iLIRKO). iLIRKO mice are born with functioning liver insulin receptor that was gradually deleted resulting in progressive hepatic and peripheral insulin resistance. Notably, peripheral insulin resistance developed as a secondary effect of the primary hepatic insulin resistance. This phenotype is consistent with the hypothesis that hyperinsulinaemia itself can cause insulin resistance in peripheral tissues.\textsuperscript{124} The progressive insulin resistance seen in iLIRKO mice was associated with hyperinsulinaemia and increased β-cell mass; while eventual failure of the β-cells to secrete enough insulin lead to uncontrolled diabetes. Admittedly, all insulin target tissues are relevant with regards to insulin resistance. But, the progressive nature of insulin resistance stresses the need to find the organ in which the genesis of insulin resistance ultimately leads to type 2 diabetes. Evidence from the study by Escribano et al. (2009) supports a hypothesis in which the liver may be the primary organ in the pathogenesis of type 2 diabetes.
1.6 Insulin Signalling

Insulin signalling through the insulin receptor regulates a wide variety of biological processes that are dependent on tissue type. In addition to the classical regulation of glucose/lipid homeostasis, insulin is also responsible for regulating cell growth and differentiation. Although animal studies with tissue specific ablation of the insulin receptor are useful in defining tissue relevance in insulin-resistant states, in reality complete insulin resistance is only seen in rare genetic diseases like leprechaunism and type A insulin resistance syndrome.125 Most cases of insulin resistance are due to a combination of factors that affect some, but not all outcomes of insulin action. To understand how “selective” insulin resistance may play a role in type 2 diabetes it is necessary to understand the molecular mechanisms behind insulin signalling.111

Intensive research into the insulin signalling pathway has elucidated a highly integrated and complex network of signalling components. Moreover, most of the major insulin signalling components are also involved in regulating signalling pathways initiated by other biological stimuli.126 The specific signalling initiated by insulin through what appear to be signalling hubs is likely mediated by multiple isoforms of the same regulatory protein.127 Although insulin can elicit many biological outcomes, the more established processes are presented below.

1.6.1 Insulin receptor

The insulin receptor (IR) is the first major component in the insulin signalling pathway. A member of the receptor tyrosine kinase superfamily,128 the IR and its close homologue the insulin-like growth factor-1 receptor (IGF-1R),129-131 are large heterodimers that consist of two α and β subunits. The α-subunit (Mr 140 kDa), which resides completely in the extracellular matrix, is linked to the intracellular β-subunit (Mr 95 kDa) via disulfide bonds. Insulin binding to the α-subunit leads to conformational changes that induce autophosphorylation of several tyrosine residues on the β-subunit.132-134 Autophosphorylation of the β-subunit activates the receptor’s protein tyrosine kinase, which activates intracellular substrates responsible for the metabolic consequences of insulin signalling.
1.6.2 Insulin receptor substrate (IRS) proteins

To date, 11 intracellular substrates of the IR have been identified,\textsuperscript{127} six of which are structurally similar and have been termed insulin receptor substrate proteins (IRS1-6).\textsuperscript{135-140} Although the IR tyrosine kinase interacts with other intracellular targets including Shc,\textsuperscript{141} Cbl,\textsuperscript{142} p62dok,\textsuperscript{143} and Gab-1,\textsuperscript{144} the IRS proteins are responsible for mediating activation of the two main pathways in insulin signalling. Namely, the phosphatidylinositol 3-kinase (PI3K)-AKT pathway and the Ras-mitogen activated protein kinase (MAPK) pathway.\textsuperscript{127} IRS-mediated signalling is particularly important in regulation of glucose homeostasis in the liver.

IRS proteins contain a pleckstrin homology (PH) domain, a protein tyrosine binding domain (PTB) and tyrosine residues that can be phosphorylated by the IR tyrosine kinase.\textsuperscript{140} Phosphorylation of IRS tyrosine residues creates recognition sites for intracellular molecules with a src-homology-2 (SH2) domain. Thus, IRS proteins function as docking molecules in order to facilitate propagation of the insulin signal throughout the cell.

The tissue distribution of IRS proteins varies considerably: IRS-1 and IRS-2 are widely distributed,\textsuperscript{145} IRS-3 has predominant expression in liver and lung tissue,\textsuperscript{146} IRS-4 is primarily expressed in embryonic tissue\textsuperscript{136} while IRS-5/6 appear to have limited tissue expression.\textsuperscript{135} Despite the high level of homogeneity between the IRS proteins, data from animal studies suggests that IRS-1 and 2 may have increased importance in glucose homeostasis. IRS-1 knockout mice have glucose intolerance and peripheral insulin resistance,\textsuperscript{147, 148} whereas IRS-2 knockout mice develop diabetes because of hepatic insulin resistance and lack of pancreatic β-cell compensatory response.\textsuperscript{149, 150} Conversely, IRS-3\textsuperscript{151} and IRS-4\textsuperscript{152} knockout mice have little to no perturbations in glucose homeostasis. These data suggest IRS function may be dependant on tissue and subcellular location.
1.6.3 Negative regulation of IR activity

Left unchecked, insulin activation of the IR would lead to extreme metabolic changes. Insulin-induced down regulation of IR is a well established feedback mechanism\textsuperscript{153-158} that regulates the strength of insulin signalling and is common in hyperinsulinaemic states.\textsuperscript{159} However, the contribution insulin-induced down regulation of the IR has to the pathogenesis of type 2 diabetes is unclear. Other mechanisms that effect negative regulation of IR signalling include: protein tyrosine phosphatase-mediated deactivation of the IR tyrosine kinase, steric blocking of IR-IRS interaction and inhibition of insulin binding to the IR.

Of the protein tyrosine phosphatases, PTP1B is the most well studied.\textsuperscript{160} PTP1B acts to dephosphorylate the IR tyrosine kinase thus terminating the insulin signal.\textsuperscript{161} Animals with liver specific deletion of PTP1B have improved insulin sensitivity and glucose homeostasis\textsuperscript{162} leading some to suggest PTP1B inhibitors as a possible treatment for type 2 diabetes.\textsuperscript{163} Similarly, suppressors of cytokine signalling-1 and 3 (SOCS-1 and 3) proteins are thought to be involved in insulin resistance through inhibition of tyrosine kinase phosphorylation of IRS proteins.\textsuperscript{164} Notably, SOCS proteins have been shown to be elevated in obesity,\textsuperscript{165} a state often associated with insulin resistance.

Of particular interest is inhibition of insulin binding to the IR α-subunit by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1 also referred to as plasma cell membrane glycoprotein 1 or PC-1).\textsuperscript{166} Increased ENPP1 protein levels have been found in skeletal muscle,\textsuperscript{167} adipose tissue\textsuperscript{168} and skin fibroblasts\textsuperscript{169, 170} from insulin-resistant individuals. Subsequently, high levels of ENPP1 coincided with decreased insulin receptor activity implicating ENPP1 in the pathogenesis of insulin resistance and type 2 diabetes.\textsuperscript{171} Overexpressing ENPP1 \textit{in vitro}\textsuperscript{170} and \textit{in vivo}\textsuperscript{172} led to impaired insulin signalling and action while selectively suppressing the levels of ENPP1 in the liver of mice improved insulin sensitivity.\textsuperscript{173} Moreover, there is compelling evidence suggesting a role for ENPP1 in genetic forms of insulin resistance. Genotype-phenotype association studies have identified a gain of function mutation (K121Q), which increases the inhibitory function of ENPP1 and may have a role in insulin resistance and type 2 diabetes.\textsuperscript{174, 175}
1.6.4 PI3-K/AKT pathway

Phosphatidylinositol 3-kinase (PI3-K) is a pivotal kinase involved in mediating metabolic outcomes of insulin signalling. It consists of a regulatory p85 and a catalytic p110 subunit, both of which occur in several isoforms. The p85 subunit of PI3-K has two SH2 domains that interact with the phosphotyrosine residues on IRS-1. Although PI3-K is involved in multiple biological processes, production of PI-3P, PI-(3,4)P_2 or PIP_2 and PI-(3,4,5,)P_3 or PIP_3, from the phosphorylation of phosphoinositides at the 3-position, are the most important in insulin signalling. PIP_2 and PIP_3 go on to bind the PH domains of other molecules thus activating them or changing their subcellular location.

Although PIP_3 regulates three main classes of signalling molecules, the AGC family of serine/threonine protein kinases is the most studied and pertinent to glucose metabolism. Of the AGC kinases, phosphoinositide-dependant kinase 1 (PDK1) is the best characterized and along with PIP_3, is involved in activating a serine/threonine kinase called AKT. PIP_3 activates PDK1, which phosphorylates and increases the catalytic activity of AKT. But, because PDK1 resides at the plasma membrane, PIP_2 and PIP_3 must first facilitate the interaction of PDK1 and AKT by recruiting it to the plasma membrane via its PH domain. Once activated, AKT is thought to mediate the majority of the hepatic metabolic outcomes stimulated by insulin.

1.6.5 PI3-K/AKT-mediated regulation of glycogen synthesis

Glucose entering the cell through the glucose channels is converted to glycogen by glycogen synthase (GS). Insulin activates glycogen synthase by promoting its dephosphorylation via inhibition of glycogen synthase kinase 3 (GSK-3). Active AKT phosphorylates and deactivates GSK-3, thus decreasing the rate of phosphorylation of GS and increasing its activity. Insulin has also been shown to target protein phosphatase 1 (PP1) which further increases the dephosphorylation and activation of GSK-3. Rather than globally activating PP1, insulin is thought to stimulate PP1 activity that is localized to glycogen particles. Although there is no direct evidence of the mechanisms behind insulin activation of PP1, inhibitors of PI3-K block this effect suggesting the involvement of the PI3-K/AKT pathway.
Figure 1.6-1. A brief depiction of insulin signalling and its outcomes in the hepatocyte. Insulin binding to the IR at the hepatocytes regulates glucose and lipid homeostasis and cell growth and differentiation. Activation of the tyrosine kinase catalyses the phosphorylation of proteins like the IRS and Shc. These proteins then interact with signalling molecules through their SH2 domains resulting in activation of PI3-K and the Ras-MAPK cascade. PI3-K activates AKT, which regulates two of the major mechanisms involved in glucose homeostasis in the liver. First, AKT phosphorylates and deactivates GSK-3, which stops it from phosphorylating GS thus increasing its activity and glycogen synthesis. Second, AKT phosphorylates FOXO-1, thus preventing it from entering the nucleus and activating transcription of gluconeogenic genes (G6P and PEPCK) thus decreasing gluconeogenesis. Finally, although the exact mechanisms are unclear, insulin increase the levels of both SREBP-1c transcript and its mature nuclear form. Nuclear SREBP-1c increases transcription of lipogenic genes thus increasing lipogenesis. IRS: insulin receptor substrate; PI3-K: Phosphatidylinositol 3-kinase; MAPK: mitogen activated protein kinase; GSK-3: glycogen synthase kinase 3; GS: glycogen synthase; FOXO-1: forkhead transcription factor 1; G6P: glucose-6-phosphate; PEPCK: phosphoenolpyruvate carboxy kinase; SREBP-1c: sterol regulatory element binding protein 1c. (adapted from Saltiel and Kahn, 2001)
1.6.6 PI3-K/AKT-mediated regulation of hepatic glucose output

Insulin regulates hepatic glucose output through direct effects on gene expression\textsuperscript{188} and indirect regulation of substrate availability.\textsuperscript{182} Insulin inhibits transcription of gluconeogenic genes that code for phosphoenolpyruvate carboxy kinase (PEPCK),\textsuperscript{189, 190} glucose-6-phosphatase\textsuperscript{191, 192} and fructose-1,6-bisphosphatase, while increasing transcription of genes that code for glycolytic enzymes such as glucokinase\textsuperscript{193} and pyruvate kinase.\textsuperscript{194} Current evidence suggests that forkhead transcription factor 1 (FOXO-1)\textsuperscript{192, 195} and the transcriptional co-activator PGC-1\textsuperscript{196} are involved in regulating gluconeogenesis in the liver. Specifically, AKT phosphorylates FOXO-1 which prevents it from entering the nucleus and activating transcription of genes that code for glucose-6-phosphatase and PEPCK, an enzyme that catalyzes the rate limiting step in gluconeogenesis.\textsuperscript{191, 197}

1.6.7 PI3-K/AKT-mediated regulation of lipid homeostasis

In addition to the regulatory action on glucose homeostasis, insulin also has a critical role in lipid homeostasis. Insulin deactivates hormone sensitive lipase (HSL) through the PI3-K/AKT pathway thus inhibiting lipolysis in adipose tissue and depriving the liver of fuel (FFA) for glucose production.\textsuperscript{198} In the liver, insulin promotes lipogenesis by increasing transcription of lipogenic genes like acetyl-CoA carboxylase.\textsuperscript{199} In the same way insulin’s effects on glucose metabolism are regulated by FOXO-1, sterol regulatory element binding protein 1c (SREBP-1c) mediates many of insulin’s effects on lipogenesis.\textsuperscript{200} Induction of SREBP-1c transcription appears to be dependant both on AKT\textsuperscript{201-203} and liver X receptor activation,\textsuperscript{204} although the exact mechanism is unknown. Unlike FOXO-1; however, SREBP-1c regulation of lipogenic gene expression is a complex process and is subject to a sterol sensing element within the cell. Insulin increases levels of SREBP-1c transcript and nuclear protein.\textsuperscript{205, 206} SREBP-1c transcripts encode a membrane bound precursor, which is held in the endoplasmic reticulum by Insig proteins. Sterol depletion causes the SREBP cleavage activating protein (SCAP) to transport SREBP-1c to the Golgi apparatus, where it undergoes cleavage by two proteases. Once cleaved, the N-terminal fragment of SREBP-1c is released and is transported to the nucleus, where it induces transcription of lipogenic genes that promote triglyceride synthesis.
1.6.8 Ras/MAPK pathway

The Ras/MAPK pathway is the other major signalling network that can be stimulated by insulin and is involved in mediating cell growth, differentiation and survival. Insulin causes tyrosine phosphorylation of IRS-1, Gab1 and Shc, thus facilitating binding of growth factor receptor binding protein (Grb-2).\textsuperscript{207} Grb2 recruits the guanyl nucleotide exchange protein (SOS) to the plasma membrane resulting in the activation of G protein Ras.\textsuperscript{207} Activated Ras induces a phosphorylation cascade that begins with Raf and ends with activation of the Mitogen activated protein kinase (MAPK)/Extracellular signal regulated kinases (ERK) pathway. The activated ERKs phosphorylate various intracellular substrates and translocate to the nucleus to phosphorylate transcription factors, like Elk-1, that promote gene expression. Studies with Ras or SOS dominant negative mutants and with IRS-1 siRNA/antibodies have shown that the Ras/MAPK pathway is important for insulin’s effect on cell growth and DNA synthesis.\textsuperscript{208-211}

1.7 Obesity and type 2 diabetes: the search for a common pathway

Genetic and environmental factors can act alone or in unison to cause insulin resistance. Hereditary diseases of insulin resistance such as leprechaunism,\textsuperscript{212} Rabson-Mendenhall syndrome\textsuperscript{213, 214} and Type A insulin resistance\textsuperscript{125} are rare and often involve mutations of the IR gene. Environmental factors such as obesity; however, are commonly associated with insulin resistance and type 2 diabetes.\textsuperscript{215} Like diabetes, obesity is a worldwide epidemic that shows no signs of abating. Recent estimates show that in the United States, more than one third of adults (35.7\%) and 17\% of children were considered obese.\textsuperscript{216} In New Zealand, 27.8\% of adults and 8.3\% of children were classified as obese by two nationwide health surveys.\textsuperscript{217, 218}

1.7.1 Obesity and β-cell dysfunction

Obesity has a complex relationship with insulin resistance and type 2 diabetes. Indeed, \~25-30\% of obese individuals remain metabolically normal retaining normal levels of insulin sensitivity, blood pressure, lipid profile and inflammatory profile.\textsuperscript{219-}
Further, many obese insulin-resistant individuals do not develop hyperglycaemia. This is because the pancreatic \( \beta \)-cells compensate for decreased insulin sensitivity by increasing insulin secretion thus maintaining physiological glucose concentrations. Type 2 diabetes is prevented as long as the \( \beta \)-cells can secrete enough insulin to overcome the insulin resistance. Increased insulin secretion from the \( \beta \)-cells appears to be mediated by both increased \( \beta \)-cell mass and enhanced \( \beta \)-cell function. However at a certain point, \( \beta \)-cells fail to produce enough insulin resulting in impaired glucose tolerance and ultimately frank hyperglycaemia. A recent review by De Fronzo and Abdul-Ghani (2011) has suggested that preservation of 20-30% of \( \beta \)-cell function may prevent individuals from progressing to type 2 diabetes.

Several lines of evidence suggest that \( \beta \)-cell failure has a genetic component. Only a subset of individuals progress to type 2 diabetes, while abnormal insulin secretion has been observed in first-degree relatives of individuals with type 2 diabetes. Longitudinal data from the Pima Indians, who have the highest prevalence of diabetes than any other group in the world, shows that progression from impaired glucose tolerance to diabetes coincided with \( \beta \)-cell failure. Although there is a genetic component, obesity and the concomitant insulin resistance have a critical role to play in diabetes progression.

### 1.7.2 Obesity and insulin resistance

The recent emergence of adipose tissue as a secretory organ has revolutionized the way researchers consider obesity and metabolic disease. Adipose tissue can influence glucose homeostasis by release of metabolites (FFA), hormones (leptin, adiponectin, resistin, visfatin) and proinflammatory cytokines (IL-1, TNF\( \alpha \), MCP-1). Consequently, obesity has been associated with an increase in the production of many of these products, with adipokine-mediated release of proinflammatory factors being implicated in insulin resistance.

Although the mechanisms behind the affect of obesity on insulin resistance are incompletely understood, it has been suggested that it may be due to the distribution of fat. Fat distribution can be generally classified as lower body, abdominal
subcutaneous (underneath the skin), overall coverage or visceral fat (located among the organs of the abdomen). Visceral fat is thought to increase the risk of insulin resistance because of its anatomical location and venous drainage of secreted factors into the hepatic portal vein. Consequently, it has been suggested that high visceral fat content leads to increased FFA deposition in the liver and insulin resistance. However, other studies have presented data to the contrary suggesting there is no direct causative link between visceral fat tissue and hepatic insulin resistance. In individuals with lipodystrophy and a fatless mouse model, hepatic insulin resistance and marked liver steatosis was present despite a lack of visceral adiposity. Although intra-abdominal fat mass has been observed to have a detrimental affect on insulin sensitivity by increasing both ectopic lipid deposition and inflammation, its exact role in mediating insulin resistance is unclear.

Obesity associated insulin resistance is often caused by external factors that interfere in the insulin signalling pathway. Several mechanisms have been implicated in the pathogenesis of type 2 diabetes, which are: glucotoxicity, lipotoxicity, oxidative stress, endoplasmic reticulum stress and inflammation. Each is a distinct mechanism and they are thought to equally contribute to the development of the disease. However, a unifying pathway that can be sequentially followed from inception of insulin resistance to type 2 diabetes has yet to be elucidated. Nonetheless, recent advances in the field have increased the significance of three of these mechanisms (lipotoxicity, ER stress and inflammation) and their contributions to development of insulin resistance.

1.7.3 Lipotoxicity

It is now generally accepted that increased levels of FFAs have a detrimental effect on insulin action as they have been shown to cause insulin resistance and impaired insulin secretion. Obese individuals with or without type 2 diabetes have increased levels of FFAs. A short term (48 hours) sustained increase of FFAs was shown to cause impaired insulin secretion in response to mixed meals and intravenous glucose. The evident toxic effects of elevated FFAs led to the coining of the term ‘Lipotoxicity’.
Lipotoxicity in muscle tissue impairs the insulin signalling cascade and insulin-mediated glucose uptake causing insulin resistance.\textsuperscript{249-251} Specifically, lipid accumulation, rather than the circulating plasma lipid concentration, is responsible for affecting insulin action.\textsuperscript{252, 253} Non-alcoholic fatty liver disease (NAFLD) is the clinical term for lipid accumulation in the liver. NAFLD can range from simple steatosis to steatohepatitis, advanced fibrosis and cirrhosis, and is commonly found in individuals with obesity and type 2 diabetes.\textsuperscript{254} Fabbrini \textit{et al.} (2009) demonstrated that intra-hepatic fat was associated with the metabolic complications of obesity.\textsuperscript{255} Reversing NAFLD with a strict hypocaloric diet in individuals with type 2 diabetes improved liver insulin sensitivity (but not peripheral) and normalized fasting hyperglycaemia.\textsuperscript{256} Similarly, a recent study suggested strict reduction of dietary energy intake could reverse the underlying abnormalities present in individuals with type 2 diabetes and insulin resistance. Notably, decreased pancreatic and liver lipid accumulation was associated with improved hepatic insulin sensitivity, insulin secretion (first phase response) and normalization of fasting hyperglycaemia.\textsuperscript{257}

\textbf{1.7.4 Molecular mechanism behind lipotoxicity}

Increased FFA delivery to tissue results in increased intracellular content of fatty acid metabolites such as diacylglycerol (DAG), fatty acyl-coenzyme A (fatty acyl-CoA) and ceramides, which can act as secondary messengers in key signalling pathways.\textsuperscript{244} Consequently, high levels of ceramides and DAG in liver and muscle tissues were associated with development of insulin resistance.\textsuperscript{258}

DAGs mediate insulin resistance via an interaction with several molecules belonging to the protein kinase C family (PKC).\textsuperscript{244, 259} The PKC family belongs to the AGC kinase superfamily and includes: conventional PKCs (cPKC: α, βI, βI I, γ), novel PKCs (nPKC: ε, θ, η, δ) and atypical PKCs (aPKC: ζ and λ).\textsuperscript{244} Specifically, DAG-mediated activation of PKC0 has been shown to cause phosphorylation of IRS-1. This in turn blocks insulin-induced phosphorylation of IRS-1 and inhibits signalling through the PI3-K/AKT pathway.\textsuperscript{260, 261} PKCε has also been shown to impair IR tyrosine kinase activity in liver tissue. Rats that were fed a high fat diet developed hepatic steatosis and hepatic insulin resistance, which was associated with increased
levels of PKCε. Subsequent knockdown of PKCε reversed the fat-induced effects on insulin signalling and protected the rats from hepatic insulin resistance.262

Evidently, DAG-mediated activation of nPKCs can impair insulin signalling and action in key tissues like the liver and muscle. Similarly, ceramide accumulation in tissue is thought to mediate insulin resistance by inhibiting activation of AKT.263, 264 Ceramide accumulates in membrane microdomains where it recruits PKCζ.265, 266 PKCζ phosphorylates AKT and prevents it being bound by PIP3.267 This inhibits the actions of insulin through the PI3-K/AKT pathway causing insulin resistance with regards to glucose metabolism.

1.7.5 Inflammation

Chronic low-grade systemic inflammation has a complex but critical role in insulin resistance.235 Initial observations of elevated acute-phase reactants and cytokines in obesity and type 2 diabetes implied that stimulation of the innate immune response had a pathological role in this disease.268-271 Elevated levels of interleukin-1β (IL-1β), IL-6 and CRP were found to be predictive of diabetes development.272, 273 Furthermore, serum concentrations of IL-1 receptor antagonist (LI-1RA) were increased in obesity274 and continue to increase in the years preceding diabetes.275 Over the last decade, obesity has been characterized by a broad array of inflammatory factors that can impact on insulin signalling.234 TNFα provided the first molecular link between obesity, inflammation and insulin resistance when it was found to be increased in adipose tissue of insulin-resistant rodents.276 Similarly, increased TNFα levels were observed in adipose tissue of obese individuals270, 271 and muscle tissue of insulin-resistant individuals with type 2 diabetes.277 Subsequent studies of the mechanism behind TNFα-mediated insulin resistance suggested that TNFα induces serine phosphorylation (Ser 307) of IRS-1 thus inhibiting insulin signalling through the IR.278, 279

Although the exact mechanisms behind obesity-induced inflammation are currently unclear, recruitment of adipose tissue macrophages (ATMs) by chemokine signalling is thought to play a key role.280-283 ATMs secrete many inflammatory factors and they were found to be responsible for most of the adipose tissue associated TNFα
secretion. Overexpression of monocyte chemo attractant protein-1 (MCP-1) in adipose tissue increased ATM infiltration leading to development of insulin resistance and hepatic steatosis; whereas expression of a dominant negative mutant of MCP-1 improved insulin resistance in db/db mice and mice fed a high fat diet. Furthermore, research over the last five years has implicated T lymphocytes, natural killer T cells, mast cells, and B cells in obesity-related insulin resistance.

However, a recent study by Meijer et al. (2011) has shown that primary adipocytes can secrete cytokines and chemokines independently of macrophages. This led the authors to suggest that adipocyte metabolic dysfunction is the primary event leading to chronic inflammation, which can be aggravated by ATM infiltration. Nonetheless, ATM infiltration was shown to be a strong predictor of insulin sensitivity in obese individuals. Collectively, these data suggest that infiltration of adipose tissue by ATMs is a part of a broader inflammatory response associated with hepatic lipid accumulation and insulin resistance.

1.7.6 Molecular mechanisms behind inflammation-mediated insulin resistance

Inflammation contributes to insulin resistance primarily through inhibition of downstream insulin signalling. Jun-terminal kinase (JNK) and inhibitor of nuclear factor-κβ kinases (IKK) are activated by stress stimuli present in metabolic disease (cytokines and FFAs) and have been implicated in insulin resistance. IKK can inhibit insulin signalling both directly and indirectly. Mice which lack IKK were partially protected from high-fat diet or lipid infusion-induced insulin resistance. Consequently, IKK was shown to inhibit insulin signalling by disrupting the IR-IRS-1 interaction via direct phosphorylation of IRS-1 on serine residues. Not surprisingly, mice with hepatic IKK knockout retain liver insulin sensitivity, although they do develop muscle and fat insulin resistance in response to a high fat diet. IKKβ also indirectly impairs insulin signalling by activating NF-κβ via phosphorylation of inhibitor of NF-κβ (Iκβ). NF-κβ is a transcription factor that stimulates production of multiple inflammatory factors, including TNFα and IL-6. Transgenic mice overexpressing IKK in liver tissue exhibited hyperglycaemia, marked hepatic insulin resistance and moderate systemic insulin resistance, which was
associated with an increased liver production of IL-6, IL-1 and TNFα. Although activation of the IKK pathway in liver tissue appears to have a critical role in obesity-induced insulin resistance, it may not be as important in muscle tissue.292, 294, 295

JNKs belong to the MAPK family and regulate transcription via activation of the activating protein-1 (AP-1) complex.296 JNK is now thought to have a central role in obesity-induced insulin resistance.297 Like TNFα and IKKβ, JNK activation by cytokines and free fatty acids leads to phosphorylation of IRS-1 which inhibits IRS-1 interaction with the IR and thus insulin signalling.279, 288, 298 Overexpressing a dominant-negative type JNK in liver of obese diabetic mice markedly improved insulin sensitivity and glucose tolerance, suggesting the JNK liver pathway may have a critical role in regulating glucose homeostasis.299 Consequently, inhibition of JNK signalling has been suggested as a viable treatment for diabetes.300

Another key inflammatory system involved in obesity-induced insulin resistance is the IL-1 pathway. Elevated circulating levels of IL-1β are associated with an increased risk of developing type 2 diabetes, whereas blockage of IL-1 signalling in individuals with type 2 diabetes improved glycaemic control and β-cell function.301 Excessive IL-1β production is thought to be regulated by FFAs,302 with recent work suggesting this effect is mediated by the inflamasome.303 The IL-1β pathway is similar to IKK in that it affects insulin signalling in multiple ways. IL-1β has toxic effects on pancreatic β-cells and has been implicated in β-cell deterioration caused by glucotoxicity in type 2 diabetes, suggesting a role in impairment of insulin secretion.304 It was found to inhibit insulin signalling in adipose cells by inducing down regulation of IRS-1 gene expression.305 Furthermore, IL-1β stimulates proinflammatory actions through the IL-1 receptor that in turn recruits the IL-1 receptor accessory protein. This receptor complex initiates a signalling cascade that results in the activation of multiple inflammatory factors, which include, JNK, IKK and AP-1, all of which have been shown to inhibit insulin signalling.306 Accordingly, animal models that lack IL-1β receptor were protected against high fat diet-induced insulin resistance.307
1.7.7 The unfolded protein response: a link between lipotoxicity and inflammation

The emergence of lipotoxicity and obesity-related inflammation has refocused the way the scientific community looks at metabolic disease and type 2 diabetes. Although we are aware of some of the major factors involved in these two processes, there are still many unanswered questions. It is interesting to note that lipid infusion or high fat diets are a common method of producing insulin resistance and type 2 diabetes in animal models. Conversely, animals that had ablation of inflammatory factors were protected against insulin resistance, while treatment of humans with drugs that target inflammatory pathways improves insulin sensitivity. The apparent intimate relationship between lipotoxicity and inflammation points to a unifying mechanism involved in mediating the pathological processes of insulin resistance. Endoplasmic reticulum (ER) stress is emerging as a key process in obesity and diabetes and may fill this role.308

The ER is responsible for protein folding, maturation and trafficking, and as such is central in many intracellular biological processes. Stressful conditions (like hyperinsulinaemia) cause overproduction of proteins, thus increasing the amount of unfolded proteins in the ER lumen, which leads to consequent initiation of the unfolded protein response (UPR).309

Activation of the UPR leads to reduced protein synthesis, increased protein degradation and production of chaperones that mediate protein folding. Insufficient reduction of ER stress by the UPR leads to compromised cell functionality and apoptosis. The actions of the UPR are mediated by three ER-membrane associated proteins that regulate three mechanistically distinct UPR pathways: PKR-like eukaryotic initiation factor 2α kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor-6 (ATF6).309 In the absence of ER stress, these three proteins are inactive due to sequestration via binding of BiP/GRP78.310 Accumulation of unfolded proteins in the ER disassociates BiP/GRP78, inducing activation of PERK and IRE1 and translocation of ATF6 to the Golgi apparatus.309

The UPR has been linked to inflammation via the JNK and IKK pathways.311, 312 During ER stress, the IRE1 arm of the UPR was shown to promote inflammatory
signalling through activation of JNK.\textsuperscript{313} Similarly, activation of the ATF6 arm of the UPR was also associated with NF-κβ activation.\textsuperscript{314} Interestingly, induction of ER stress in macrophages increased levels of proinflammatory factors such as TNFα and IL-6, which was shown to be mediated by free cholesterol activation of the IKK and JNK pathways.\textsuperscript{315} Activation of these inflammatory pathways and induction of both cytokines was reliant on cholesterol trafficking to the ER, suggesting a possible link between lipotoxicity and UPR-mediated inflammation.

Current data suggests the ER is an integral organelle in the regulation of glucose and lipid metabolism. Hepatic gluconeogenesis was shown to be regulated by CREBH, an ER-bound transcription factor.\textsuperscript{316} Consequently, animal models have shown that disrupting the PERK pathway of the UPR resulted in hypoglycaemia associated with defective gluconeogenesis.\textsuperscript{317, 318} Interestingly, inhibiting PERK signalling in mice reduced insulin sensitivity in muscle and adipose tissue, suggesting a possible crosstalk mechanism between the liver and peripheral insulin target tissue.\textsuperscript{319}

ER-mediated regulation of lipid homeostasis is emerging as a possible link between lipotoxicity and insulin resistance. As discussed previously, stimulation of lipogenesis occurs via insulin-mediated SREBP-1c protein activation, which resides on the ER.\textsuperscript{320} Interestingly, inhibiting the UPR in liver tissue inhibited SREBP-1c cleavage and expression, thus reducing hepatic triglyceride and cholesterol levels and improving insulin sensitivity.\textsuperscript{321} The effects of the UPR on lipid metabolism are thought to be regulated by IRE1α and ATF6-mediated pathways. IRE1α represses expression of key metabolic enzymes, including those involved in triglyceride biosynthesis and lipid secretion.\textsuperscript{322} Although mice that have complete ablation of liver IRE1α are not significantly different from wild type littermates, they do develop profound lipid accumulation after induction of ER stress with chemical agents.\textsuperscript{322} Similarly, ATF6 knockout mice are phenotypically normal until chemical induction of ER stress causes lipid droplet accumulation and hepatic steatosis.\textsuperscript{323} Taken together, these studies suggest that the actions of the UPR are critical in regulating glucose and lipid homeostasis.

However, there is no clear consensus on whether the UPR directly interferes with insulin signalling or is simply a cellular response to metabolic changes.\textsuperscript{244} Nonetheless, ER stress has been implicated in obesity-related insulin resistance in
animal models and humans. Insulin resistance in leptin-deficient ob/ob mice was associated with activation of the UPR, which led to inhibition of insulin of signalling via JNK-mediated phosphorylation of IRS-1.\textsuperscript{324, 325} Similarly, reduction of ER stress was observed in individuals who had substantial weight loss after bariatric surgery.\textsuperscript{326} Consequently, treating obese mice that had type 2 diabetes with chemical agents that reduce ER stress normalized hyperglycaemia, restored insulin sensitivity and resolved NAFLD.\textsuperscript{327} A similar effect was observed in humans,\textsuperscript{328} although others have suggested that these agents have confounding effects on lipogenesis\textsuperscript{329} and thyroid hormone regulation of energy expenditure.\textsuperscript{330}

Although there is compelling evidence supporting an important role for the UPR in insulin resistance, much is still not known regarding the three key pathway of the UPR. Evidently, certain components of the UPR regulate lipid (IRE1α and ATF) and glucose metabolism (PERK), while activation of the UPR may have role in NAFLD.

1.8 A potential model detailing progression from insulin resistance to type 2 diabetes

An important feature of type 2 diabetes and its associated complications is the progressive nature of the disease. A brief look at some of the mechanisms behind the factors involved in insulin resistance reveals several feed-forward mechanisms that become progressively exacerbated as time passes on.

1.8.1 Lipotoxicity and inflammation: a feed-forward mechanism

FFAs and other metabolites like glucose are thought to have a role in activating the inflamasome.\textsuperscript{303} Several studies have demonstrated FFA activation of toll like receptors,\textsuperscript{331, 332} which can induce production of NF-κβ and other inflammatory factors.\textsuperscript{333} Treatment of macrophage cells with palmitate (FFA) was shown to induce expression of TNFα and IL-1β among others.\textsuperscript{334} Many of these cytokines can further activate the JNK and IKK pathways in a feed-forward manner, thus exponentially increasing the effect of the inflammatory response.
On the other hand, several cytokines were observed to have a lipolytic effect in adipose tissue thus increasing circulating FFAs. Although inflammation is thought to have a regulatory role in lipid homeostasis, the underlying mechanisms are unclear. IL-6 and TNFα were shown to stimulate lipolysis in adipose tissue\textsuperscript{335-338} and while IL-6 was shown to increase hormone sensitive lipase (HSL) mRNA expression, it did not have an effect on HSL protein activity.\textsuperscript{337} TNFα is also thought to indirectly promote lipolysis by decreasing expression of perilipin (PLIN), a protein involved in forming lipid droplets that protect triglycerides from lipases.\textsuperscript{339-341} Accordingly, lack of PLIN expression was associated with markedly increased lipolysis.\textsuperscript{342, 343} Recently, a newly identified lipid droplet associated protein, fat specific protein 27 (FSP27), was found to be down regulated by TNFα, IL-1β and IFNγ.\textsuperscript{344} Like PLIN, FSP27 is a lipid droplet stabilizer and decreasing its expression mirrors the lipolytic effects of TNFα.\textsuperscript{344} Taken together, these data suggest a self-feeding cycle that leads to exacerbated lipotoxicity and inflammation.

The development of insulin resistance thereafter appears to be a matter of time and although the exact sequence of events that lead to type 2 diabetes are yet to be determined, several animal models provide clues as to the possible mechanisms.

\textbf{1.8.2 Primary hepatic insulin resistance in animal models}

Recently, several experiments using microarray technology have confirmed variable hepatic gene expression consistent with a key role of inflammation in high fat diet-induced insulin resistance. In insulin-resistant, hyperglycaemic mice, genes that regulate inflammation were up regulated early and were consistently elevated. Additionally, variable expression of genes that regulate lipid homeostasis were concomitant with dysregulated lipid metabolism.\textsuperscript{345-347} Notably, Kleeman \textit{et al.} (2010) used the same method to investigate variable gene expression in liver, muscle and fat tissue in a time resolved manner.\textsuperscript{348} In mice fed a high fat diet there was a rapid up regulation of genes involved in carbohydrate (only liver tissue) and lipid (liver and fat tissues) metabolism, whereas these genes were gradually down regulated in skeletal muscle tissue.\textsuperscript{348}
Interestingly, Kleeman *et al.* (2010) observed that a high fat diet-induced insulin resistance primarily in the liver (week six) and then in adipose tissue (week 12), while skeletal muscle remained sensitive. The mechanisms behind this are currently unclear although inflammation in adipose tissue is thought to shift toward the liver resulting in hepatic steatosis.\textsuperscript{349} Recent evidence presented by Nov *et al.* (2013) has suggested a possible IL-1\(\beta\) crosstalk mechanism between adipose tissue and the liver.\textsuperscript{350} IL-1\(\beta\) levels were elevated preferentially in portal blood of high-fat diet mice and were also linked to regulation of hepatic gluconeogenic flux.

The manifestation of early hepatic insulin resistance and subsequent peripheral insulin resistance is consistent with animal studies done by Kahn and colleagues.\textsuperscript{116, 123} Despite the methodological differences (hepatic IR ablation versus high-fat diet), in both cases primary hepatic insulin resistance seems to be a precursor for development of whole body insulin resistance. A recent study on the pathological properties of hepatic ER stress revealed a possible role for the UPR in mediating cross talk between the liver and peripheral organ insulin sensitivity.\textsuperscript{319}

Mice fed a high fat diet developed hepatic steatosis and insulin resistance that was associated with augmented signalling through the PERK arm of the UPR. Inhibition of PERK arm signalling decreased hepatic glucose production, but was also associated with impaired insulin sensitivity in muscle and adipose tissue. This was suggested to be partially due to increased levels of circulating IGFBP-3.\textsuperscript{319} Interestingly, iLIRKO mice, which develop peripheral insulin resistance in muscle tissue after primary hepatic insulin resistance due to IR ablation, also had elevated levels of IGFBP-3.\textsuperscript{123} Collectively, these data suggest that a compensatory mechanism that attempts to control the abnormal production of glucose in the liver may be inadvertently causing peripheral insulin resistance in other tissue via IGFBP3.
1.8.3 The liver as a key organ in the progression to type 2 diabetes

Insulin signalling at the liver may be a principal process involved in the development of type 2 diabetes. In 1992, Dennis McGarry* was the first to suggest that hyperinsulinaemia can cause peripheral insulin resistance due to over-stimulated lipogenesis at the liver. McGarry hypothesized that overproduction of insulin by the pancreas may cause hyperinsulinaemia that drives hepatic lipogenesis and VLDL synthesis. Consequently, the increased flux of triglycerides from the liver would accumulate in muscle tissue causing insulin resistance and mild hyperglycaemia. This would further stimulate insulin secretion from the pancreas causing even greater hyperinsulinaemia eventually leading to β-cell failure and diabetes. More than a decade later, our understanding of the pathogenesis of type 2 diabetes has increased considerably and McGarry’s opinion that lipid homeostasis has a crucial role in type 2 diabetes turned out to be prophetic.

The fundamental characteristic behind McGarry’s hypothesis is the multi-stimulatory nature of insulin action. The ability of insulin to regulate glucose homeostasis, lipid homeostasis (particularly at the liver), and cell growth naturally led to the concept of “selective insulin resistance”. Several animal models have provided evidence for the importance of selective insulin resistance in type 2 diabetes. Lipodystrophic and ob/ob mice, which have hyperglycaemia and hyperinsulinaemia, were found to be insulin resistant in the FOXO-1 pathway. However, insulin sensitivity was maintained in the SREBP-1c pathway, which caused increased fatty acid synthesis and triglyceride accumulation in the liver.

The LIRKO mouse, characterized by absolute ablation of hepatic insulin signalling through the IR, is a prime example of selective insulin signalling. In these mice, insulin cannot suppress gluconeogenesis leading to hyperglycaemia, but by the same token insulin also cannot activate SREBP-1c resulting in low levels of hepatic triglycerides and circulating VLDL. Interestingly, the hyperglycaemia seen in LIRKO mice resolves with age due to increased β-cell hyperplasia. The absence of diabetes in

* Dennis McGarry (1940-2002), PhD, was a scientist who made a series of groundbreaking contributions to mammalian metabolism. His seminal paper published in Science entitled “What if Minkowski Had Been Ageusic?” revolutionized how researchers think about type 2 diabetes and led to the eventual identification of lipotoxicity as a key process in insulin resistance.
these mice was suggested to be due to the lack of lipotoxic effects on the pancreatic β-cell caused by the non-existent insulin-mediated lipogenesis at the liver.\textsuperscript{351} β-cell failure is thought to be critical in obesity-related progression to type 2 diabetes.\textsuperscript{215} Although the mechanisms governing this process are currently an active field of research a definitive mechanism that leads to β-cell failure is yet to be identified. Inflammation,\textsuperscript{235} lipotoxicity,\textsuperscript{248, 353} ER stress\textsuperscript{354} and glucotoxicity\textsuperscript{355, 356} have all been implicated in impairing β-cell function.

\subsection*{1.8.4 From insulin resistance to type 2 diabetes: a potential hypothesis}

Taken together, the evidence presented over the last 30 years allows us to form a potential hypothesis that explains the pathology behind type 2 diabetes (Figure 1.8-1). Increased levels of metabolites like lipids (lipotoxicity) and glucose (glucotoxicity), often seen in obesity, lead to inflammation in adipose and other insulin sensitive tissue (Figure 1.8-1A). Lipotoxicity and inflammation feed into each other increasing the potential for ectopic lipid deposition and exacerbation of the immune response, both of which have been implicated in mediating hepatic insulin resistance. Selective hepatic insulin resistance leads to increased lipotoxicity through over-stimulated lipogenesis and initiation of peripheral insulin resistance resulting in development of periodic hyperglycaemia (Figure 1.8-1B).
Figure 1.8-1. A hypothesized model describing the sequence of events leading to development of Type 2 diabetes. (A) Increased levels of metabolites like lipids (lipotoxicity) and glucose (glucotoxicity) lead to inflammation in adipose and other insulin sensitive tissue. (B) Selective hepatic insulin resistance leads to increased lipotoxicity through over-stimulated lipogenesis and initiation of peripheral insulin resistance resulting in development of periodic hyperglycaemia. (C) This accelerates insulin secretion from the pancreas causing further lipotoxicity and inflammation and initiating a cycle of progressive hyperinsulinaemia. (D) Ultimately, the β-cells fail resulting in frank diabetes.
This accelerates insulin secretion from the pancreas causing further lipotoxicity and inflammation and initiating a cycle of progressive hyperinsulinaemia (Figure 1.8-1C). Symptoms of metabolic syndrome like hypertension and dyslipidemia start to develop resulting in secondary pathologies like cardiovascular disease. Eventually the pancreatic β-cells start to fail leading to development of abnormal glucose tolerance (glucotoxicity) which with the combination of lipotoxicity and inflammation impairs β-cell function. Ultimately, this results in postprandial and fasting hyperglycaemia and frank diabetes (Figure 1.8-1D). In conclusion, selective insulin resistance observed at the liver leads to impaired regulation of glucose homeostasis and over-stimulation of lipogenesis and may be central to the progressive nature of type 2 diabetes. Investigating the selective nature of insulin signalling at the hepatocyte is critical to understanding this disease.

1.9 Insulin receptor isoforms and their role in selective insulin signalling

Identifying the regulatory steps at which insulin signalling pathways diverge is needed for a complete understanding of the pathogenesis of type 2 diabetes. Even though major strides have been made in understanding the mechanism behind the initiation of insulin resistance, there is still a lack of understanding of how insulin affects its broad array of signalling. Insulin’s ability to affect different outcomes in specific tissue is likely due to the many isoforms of key molecules in the insulin signalling network. Although post-receptor signalling is currently under extensive investigation by the scientific community the possibility of selective signalling through the IR isoforms has been neglected. Considering the only aspect of the insulin signalling pathway unique to insulin action is the IR, a closer look at the two isoforms of the molecule is warranted.

The IR gene consists of 22 exons and 21 introns located on chromosome 19. Alternative splicing of exon 11 generates two different protein isoforms; IR-A (without exon 11) and IR-B (with exon 11). Exon 11 consists of 36 bp that code for a 12 amino acid insert (residues 717-728) in the C terminus of the hormone binding α-subunit.
Alternative splicing of exon 11 is an evolutionary conserved process in mammals and is likely a major component of insulin-IR mediated metabolic signalling.\textsuperscript{359} Splicing of the IR is thought to occur through the inclusion/exclusion of alternative exons model.\textsuperscript{360, 361} Regulatory elements that enhance or silence exon inclusion/exclusion are often present in the alternatively spliced exon or in the flanking introns.\textsuperscript{362} These regulatory elements interact with \textit{trans}-acting proteins that cause exon inclusion/exclusion, with differences in levels and activity of these proteins resulting in tissue specific alternative splicing.\textsuperscript{362-364}

Recently, advances have been made in understanding the mechanisms regulating alternative splicing of the IR pre-mRNA. Regulatory sequences were found in intron 10 and exon 11 of the IR and have been shown to mediate both positive and negative regulation of IR splicing.\textsuperscript{360, 361} SRp20 and SF2/ASF were shown to bind enhancing sequences on exon 11, while CUG-BP1 was shown to bind silencing sequences on the same exon. Consequently, \textit{in vitro} overexpression of SRp20 and SF2/ASF increased exon 11 inclusion and IR-B expression, whereas overexpressing CUG-BP1 caused exon 11 skipping and increased IR-A expression. Although the signalling significance of exon 11 is unknown, alternative splicing of the IR varies in different tissues and diseases.

IR is expressed in a wide variety of tissues, with highest concentrations in insulin target tissue. The two IR isoforms are usually co-expressed at varying levels that depend on tissue type. In insulin target tissues such as liver, muscle and fat, IR-B expression is considerably higher than IR-A expression.\textsuperscript{365} The relative IR-B:A expression ratio in human liver was previously shown to be 9.8, while adipose and muscle cells have relative ratios of 3.18 and 1.15 respectively.\textsuperscript{366} Conversely, IR-A expression predominates in developmental tissue such as fetal cells\textsuperscript{367} and in pathological conditions like cancer.\textsuperscript{368-370}
1.9.1 Metabolic vs mitogenic outcomes of IR signalling

The IR isoforms show functional differences with regard to insulin binding, internalization and signalling. This is not surprising. The predominant expression of IR-B isoform in metabolically active tissue suggests that insulin signalling through IR-B is primarily metabolic; whereas predominant expression of IR-A in developmental and cancerous tissue suggest a strong mitogenic component. Although the different functions of the IR isoforms are generally accepted, the underlying molecular mechanisms are still unclear.

Early work on the functional characteristics of the two isoforms showed that IR-A has a twofold higher affinity for insulin than the IR-B isoform. IR-A was also found to have a faster insulin internalization rate. Indeed, data suggests that IR-A may be the primary isoform involved in CEACAM-1-mediated internalization of the insulin-IR complex. Co-expression of IR-A and CEACAM-1 in NIH 3T3 fibroblasts significantly increased insulin internalization and degradation in comparison to cells co-expressing IR-B and CEACAM-1. On the other hand, IR-B isoform was shown to have stronger activation of the receptor tyrosine kinase suggesting it is more efficient at propagating the insulin signal throughout the cell.

Kosaki and colleagues (1993 and 1995) were the first to provide a genuine link between IR-B isoform and metabolic signalling. In a series of experiments they showed that dexamethasone-treated Hep G2 cells had a switch in isoform expression towards IR-B. The increase in IR-B expression was associated with increased insulin sensitivity for insulin-mediated glucose metabolism and gene expression.

Mitogenic signalling through IR-A isoform was first evidenced by its high affinity binding of insulin like growth factor II (IGF-II). IGF-II is a growth promoting hormone that is highly homologous to insulin like growth factor I (IGF-1) and insulin, all of which can signal through the IR and IGF-1R. IGF-II binding to the IR has been shown to stimulate cell proliferation during mouse embryonic development. Consequent studies investigating IGF-II interaction with the IR isoforms demonstrated that IGF-II bound IR-A with an affinity close to that of insulin and stimulated cell growth and proliferation.
These data are consistent with predominant expression of IR-A in developmental
tissue. Indeed, IR-A interaction with the GLUT2 channel may be necessary for
glucose uptake in neonatal hepatocytes, suggesting IR-A mediates growth
promoting effects in early development.

Recently, it has been shown that proinsulin can also bind IR-A with a high affinity,
thus stimulating the mitogenic pathway through ERK/p70s6K activation. Although
proinsulin showed low metabolic signalling through IR-A, it was almost as equipotent
as insulin in stimulating cell proliferation. Given the ability of IGF-II and
proinsulin to stimulate mitogenic pathways through IR-A, it is not surprising that
increased IR-A expression has been observed in several cancers. IR-A expression has
been shown to be increased in breast cancer, ovarian cancer, leiomyosarcoma
and thyroid cancer.

As tissues age the pattern of IR isoform expression switches from IR-A to IR-B,
particulary in insulin sensitive tissue. Although IR-A is still expressed in adult tissue,
the exact mechanisms behind differential signalling through the two IR isoforms are
yet to be completely elucidated. Nonetheless, several studies have given insight into
the possible roles of IR isoforms in insulin target tissue. In the pancreas, insulin
signalling through the IR-A was found to induce transcription of the insulin gene
through the PI3-K/p70s6K pathway; whereas insulin signalling through IR-B-induced
transcription of β-glucokinase through the PI3-K/AKT pathway. The same group
went on to suggest that the molecular basis for the selective signalling may be due to
localization of the two isoforms to different areas of the plasma membrane, based on
the presence or absence of exon 11.

Recently, Giudice et al. (2011) suggested that differential signalling is dependant on
the internalization and localization of the IR isoforms. Using confocal and structured
illumination microscopy, they showed that internalization of the insulin-IR-A
complex is significantly faster than that of the insulin-IR-B complex. Moreover, it
was observed that IR-A endocytosis was associated with stronger activation of the
ERK proteins and consequent greater stimulation of gene transcription through the
AP-1 complex. Conversely, IR-B internalization was slower and was associated with
greater activation of AKT in comparison to IR-A.
Ligand-receptor complexes have been shown to still be able to phosphorylate downstream mitogenic effectors from endosomes within the cell. This has led some to suggest that insulin’s mitogenic signalling may occur through the IR-A isoform once it has been internalized into the cell. Blocking IR internalization results in inhibition of the Ras/MAPK pathway with no effect observed on IR autophosphorylation or IRS/AKT activation. Taken together, these data suggest a possible mechanism behind the selective signalling through the IR isoforms: IR-A has a faster internalization rate thus leading to greater induction of the mitogenic pathway from within the cell. In contrast, the extended presence of IR-B at the cell surface leads to stimulation of the PI3-K/AKT pathway and regulation of metabolic homeostasis.

1.9.2 Hybrid receptors

The regulation of insulin signalling is further complicated by the formation of hybrid receptors (HRs). Due to the homologous nature of insulin and IGF-1 receptors, cells expressing both receptors can form HRs between the IR isoforms and IGF-1R. The formation of HRs is thought to occur in the endoplasmic reticulum and is governed by a random assembly pattern that is dependent on the relative abundance of the two receptors. Although the biological significance of the HRs is yet to be revealed, several studies have identified ligand-hybrid receptor interactions and their possible role in disease. Initially, IR/IGF-1R receptors were shown to bind IGF-I and IGF-II with a high affinity and insulin with a low affinity (Figure 1.9-1). Further isoform specific investigations showed that IR-A and IR-B could form HR’s with equal efficiency. IR-A/IGF-1R receptors were able to bind to both IGFs and insulin, whereas IR-B/IGF-1R receptors had high binding affinity for IGF-I, a low affinity for IGF-II, and an insignificant interaction with insulin (Figure 1.9-1).
Figure 1.9-1. Schematic diagram of the different insulin receptor hybrids and ligands. IR-A has a faster internalization rate thus leading to greater induction of the mitogenic pathway from within the cell. In contrast, the extended presence of IR-B at the cell surface leads to stimulation of the PI3-K/AKT pathway and regulation of metabolic homeostasis. IR-A homodimers have a high affinity for both insulin and IGF-II, whereas IR-B homodimers only have a high affinity for insulin. Although IR-A/IR-B heterodimer receptors retain their affinity for insulin, they gain an increase in affinity for IGF-II that is comparable to IR-A homodimers. IR-A/IGF-1R hybrid receptors bind to both IGFs and insulin, whereas IR-B/IGF-1R hybrid receptors have a high binding affinity for IGF-I, a low affinity for IGF-II, and an insignificant interaction with insulin (adapted from Jensen and DeMeyts, 2009).

Similarly, IR isoforms have also been shown to form hybrid receptors (i.e. IR-A/IR-B). In the same way IR/IGF-1R hybrids have high affinity for growth promoting ligands, so do IR isoform hybrids. Although IR-A/IR-B receptors retain their affinity for insulin, they gain an increase in affinity for IGF-II that is comparable to IR-A homodimers (Figure 1.9-1). Because hybrid receptor formation increases the affinity for growth promoting ligands like IGF-II, they have been implicated in carcinogenesis. By the same token, due to the ligand binding properties of hybrid receptors, their role in insulin resistance and type 2 diabetes has been investigated, but with inconclusive results.
Although some have suggested increased IR/IGF-1R formation in adipose and skeletal muscle tissue is associated with type 2 diabetes, others have shown that hybrid receptor overexpression is not seen in individuals with insulin resistance. Nonetheless, increased formation of hybrid receptors that reduce the number of insulin binding sites may have a deleterious effect on insulin signalling and cannot be discounted.

1.9.3 Insulin receptor and its isoforms in diabetes: an incomplete story

The role of IR protein concentration in type 2 diabetes is unclear. It is generally accepted that chronic hyperinsulinaemia can induce IR downregulation. Ramos et al. (2006) demonstrated that Grb10 mediates insulin-induced IR downregulation via the proteasomal degradation pathway. Accordingly, IR content was found to be decreased in several diseases associated with hyperinsulinaemia like insulinoma and myotonic dystrophy type 1 (DM1). Obese individuals who underwent bariatric surgery had an increase in IR protein content concomitant with a decrease in fasting plasma insulin and an improvement in insulin sensitivity. However, whether insulin-induced IR downregulation is a feedback mechanism to compensate for hyperinsulinaemia or has a pathological role in insulin resistance is yet to be elucidated.

Altered expression of the two IR isoforms in key metabolic tissues may also contribute to the pathogenesis of insulin resistance and type 2 diabetes. Considering the important role of alternative splicing in regulating biological process, it is not surprising that it may have a role in disease. It has been suggested that 50-60% of mutations involved in hereditary disease affect the splicing mechanism. Although aberrant IR splicing has been implicated in other diseases, the regulation of IR splicing and its role in insulin resistance and type 2 diabetes is controversial. Several studies have associated increased IR-B expression in muscle and adipose tissues with insulin resistance and type 2 diabetes. Conversely, some have noted a marked increase in relative IR-A expression in muscle tissue of an individual with type 2 diabetes, whereas others have shown there to be no difference in expression of IR isoforms in muscle and adipose tissue between individuals with and without type 2 diabetes or obesity.
Evidently, there is no clear consensus for the role of IR splicing in type 2 diabetes, which may be due to variation in methodology and/or variability in disease progression.

Even so, increased expression of IR-A isoform has been linked with insulin resistance in individuals with DM1. Cells cultured using skeletal muscle from individuals with DM1 had decreased insulin sensitivity with respect to insulin-induced glucose uptake and glycogen incorporation. Interestingly, other muscular disorders such as Duchenne muscular dystrophy (which does not exhibit hyperinsulinaemia or insulin resistance), limb girdle muscular dystrophy and facioscapulohumeral muscular dystrophy do not show aberrant IR splicing. These data suggest a link between hyperinsulinaemia and increased expression of IR-A isoform. Furthermore, elevated expression of IR-A in cancer and a rising awareness of the link between hyperinsulinaemia and carcinogenesis provides circumstantial evidence for a relationship between hyperinsulinaemia and increased IR-A expression. Considering the different functional characteristics of the two IR receptor isoforms, their roles in the pathogenesis of type 2 diabetes still remain to be defined. In particular, there is little data on the regulation of IR splicing at the liver, a key organ in regulating insulin action.

1.10 Bariatric surgery: A model of type 2 diabetes remission

Type 2 diabetes has long been considered a chronic and incurable disease. However, almost 50 years ago it was first reported that gastrectomy could improve and in some cases induce remission of diabetes. In 1995, Pories and colleagues observed remission of type 2 diabetes in 146 morbidly obese individuals within six days of gastric bypass surgery, which was sustained for up to 14 years. It is now generally accepted that bariatric surgery, a type of gastrointestinal surgery originally designed to induce weight loss, is an effective treatment for type 2 diabetes. So much so that the IDF has recommended bariatric surgery as an option for treating morbidly obese individuals with the disease.
Traditionally, bariatric surgery has been divided into three separate categories: 1) restrictive procedures that cause weight loss by limiting food intake, 2) malabsorptive procedures that cause weight loss by interference with digestion and absorption and 3) mixed surgery procedures that are a combination of the restrictive and malabsorptive procedures. Although there are many different types of bariatric procedures, three are typically used worldwide. These include, laparoscopic adjustable gastric banding (LAGB), bilio-pancreatic diversion (BPD), and the Roux-en-Y gastric bypass (RYGB). Although other procedures like the duodenal-jejunal bypass (DJB), ileal interposition and sleeve gastrectomy are becoming increasingly popular, the LAGB and RYGB procedures are the most common.

The LAGB is an example of a restrictive surgical procedure and is relatively simple when compared to other types of bariatric surgery. In this procedure the superior portion of the stomach is encircled with a saline-filled band, which restricts entry of food into the stomach and thus causes early satiety and weight loss (Figure 1.10-1A). The BPD, however, is complex and involves manipulation of the digestive tract. The BPD causes food to bypass the small intestine (including the duodenum, jejunum and a portion of the proximal ileum) in addition to diverting bile and pancreatic secretions to the terminal segment of the ileum (Figure 1.10-1B). This ensures that bile and food are only mixed in the last 50-100 cm of the small bowel thus causing drastically reduced nutrient adsorption.

![Figure 1.10-1. Typical bariatric surgical procedures. (A) Laparoscopic adjustable gastric band (LAGB). (B) Bilio-pancreatic diversion (BPD). (C) Roux-en-Y gastric bypass (RYGB). Adapted from Dixon (2012).](image-url)
The RYGB is a mixture of procedures and involves restriction of the stomach volume and bypass of the excluded stomach and some small bowel (Figure 1.10-1C). A surgical stapler is used to reduce the stomach volume to a < 30cc gastric pouch. This pouch is completely separate from the remainder of the stomach and is anastomosed to the jejunum in a Roux-en-Y fashion. The remainder of the bowel is reconnected to the alimentary limb via an entero-entero anastomosis. Consequently, the RYGB procedure causes food to bypass ~95% of the stomach, and a significant portion of the small bowel (including the duodenum and a short portion of the jejunum).431, 432

1.10.1 Clinical outcomes of bariatric surgery

Traditional bariatric surgeries have been repeatedly observed to cause reduction in cardiovascular, cancer and diabetes-related mortality.433-438 However, there has been some controversy in the literature with regards to the quality of the data linking bariatric surgery and improvement in diabetes. Lack of randomized controlled trials and differences in methodologies (different types of bariatric surgery performed), severity of diabetes and measurement of glycaemic parameters have made direct comparisons of studies problematic.

Nonetheless, several systematic reviews and meta-analysis have shown a strong association between remission of type 2 diabetes and bariatric surgery. A systematic review of the Cochrane database concluded that surgery is more effective than conventional treatment for weight loss and was associated with improvement in type 2 diabetes and dyslipidaemia.439 Another series of systematic reviews and meta-analysis provided similar conclusions.13, 14 The first meta-analysis was published in 2004 and involved 134 studies and 22,094 individuals in which remission of diabetes was defined as persistent normoglycemia without diabetic medications.13 With these parameters and other biochemical measures, Buchwald and colleagues reported a 77% overall remission rate of type 2 diabetes after bariatric surgery (Table 1-2).
<table>
<thead>
<tr>
<th></th>
<th>LAGB</th>
<th>RYGB</th>
<th>BPD</th>
<th>Total population</th>
</tr>
</thead>
<tbody>
<tr>
<td>%EWL</td>
<td>47%</td>
<td>62%</td>
<td>70%</td>
<td>61%</td>
</tr>
<tr>
<td>Remission of type 2 diabetes</td>
<td>48% (56%)</td>
<td>84% (80%)</td>
<td>98% (95%)</td>
<td>77% (78%)</td>
</tr>
<tr>
<td>Resolution of hypertension</td>
<td>43%</td>
<td>68%</td>
<td>83%</td>
<td>61%</td>
</tr>
<tr>
<td>Improvement of hyperlipidemia</td>
<td>59%</td>
<td>97%</td>
<td>99%</td>
<td>79%</td>
</tr>
<tr>
<td>Improvement of hypercholesterolemia</td>
<td>78%</td>
<td>95%</td>
<td>87%</td>
<td>71%</td>
</tr>
<tr>
<td>Improvement of hypertriglyceridemia</td>
<td>77%</td>
<td>91%</td>
<td>100%</td>
<td>82%</td>
</tr>
</tbody>
</table>


There was also a drastic improvement in nonglycaemic effects with approximately 61% of individuals resolving hypertension while 79% had an improvement in hyperlipidaemia (Table 1-2). However, there are some caveats to this meta-analysis as most of the studies included were retrospective and typically had a follow up of 1-3 years. The second meta-analysis by Buchwald et al. (2009), which was an update on the earlier one, included 621 studies with 135,246 individuals and had a similar overall diabetes remission rate of 78%, while 62% remained in remission after 2 years of surgery (Table 1-2). The Swedish Obese Subjects (SOS) study is an ongoing (up to 20 years) case control study that has high quality long term data on individuals who had bariatric surgery and matched obese controls that underwent regular therapy. The surgery group had lower incidence rates of diabetes and hypertriglyceridemia at both the 2 year and 10 year follow up, in addition to having sustained weight loss and decreased mortality.

Until very recently, only one randomized control trial (RCT) had investigated the efficacy of bariatric surgery versus conventional treatment for type 2 diabetes. This RCT compared the LAGB to standard therapy in 60 individuals with BMI values of 30-40 kg/m².
Remission of type 2 diabetes (defined by a fasting glucose level $< 7 \text{ mmol/l}$ and an HbA1c $< 6.2$) was observed in 73% of individuals who had LAGB and standard therapy versus 13% of individuals who received standard therapy alone. In 2012, the results of two RCT provided further evidence that bariatric surgery can be more effective than conventional or intensive treatment alone.

In the first RCT, individuals with a history of diabetes ($\geq 5$ years) or glycated hemoglobin $\geq 7.0\%$ were randomly assigned to receive conventional medical therapy or undergo RYGB or BPD. Remission of type 2 diabetes was defined as a fasting glucose $< 6.5 \text{ mmol/l}$ and a glycated hemoglobin of $< 6.5\%$ in the absence of diabetic medication. At 2 years, 75% of individuals in the RYGB group and 95% of individuals in the BPD group had remission of diabetes. Conversely, no individuals in the conventional medical therapy group had remission of diabetes. In the second RCT, Schauer et al. (2012) investigated the efficacy of RYGB or sleeve gastrectomy versus intensive medical therapy alone in 150 obese individuals with uncontrolled type 2 diabetes. After 1 year, the primary endpoint (glycated hemoglobin $\leq 6\%$) was achieved in 12% of individuals in the medical therapy group versus 37% in the sleeve gastrectomy group and 42% in the RYGB group. The RCT studies by Mingrone et al. (2012) and Schauer et al. (2012) present strong evidence for the superiority of bariatric surgery in treating diabetes and its symptoms over medical therapy alone.

### 1.10.2 Defining the normalization of glucose homoeostasis after bariatric surgery

The phenomenal outcomes of bariatric surgery have lead to controversy within the scientific community as to what qualifies as a cure for a chronic disease like diabetes. Due to the lack of long-term data and level 1 evidence some authors have questioned the permanency of glycaemic normalization after bariatric surgery. Furthermore, terms like “resolution” or “remission” have been used subjectively and defined by variable glycaemic measures by different authors.

Because of the exponential increase in articles exploring bariatric surgery as a treatment for type 2 diabetes, a consensus article by Buse et al. (2009) sought to clarify the definition of treatment outcomes (Table 1-3).
Table 1-3. Treatment outcomes for type 2 diabetes as defined by Buse et al. (2009).

<table>
<thead>
<tr>
<th>Remission</th>
<th>HbA1c</th>
<th>Fasting plasma glucose</th>
<th>Treatment</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partial</td>
<td>&lt;6.5%</td>
<td>5.6-6.9 mmol/l</td>
<td>No drug treatment or ongoing procedures</td>
<td>≥ 1 year</td>
</tr>
<tr>
<td>Complete</td>
<td>&lt;6.5%</td>
<td>&lt;5.6 mmol/l</td>
<td>No drug treatment or ongoing procedures</td>
<td>≥ 1 year</td>
</tr>
</tbody>
</table>

The authors recommend the term “remission” be reserved for individuals who normalize glucose homeostasis for at least 1 year without ongoing drug treatment or procedures. They also suggested that prolonged complete remission (≥ 5 years) may be considered a “cure” or “resolution” of type 2 diabetes. However, as the 5 year period was arbitrarily chosen due to lack of long term data, the authors acknowledged the risk of relapse in a subset of the population. Consequently, the terms “remission” and “resolution” will be used in this thesis as defined by Buse et al. (2009).445

1.10.3 Weight-independent anti-diabetic effects of RYGB surgery

Particular bariatric procedures have a higher rate of diabetes remission. This is evident in Buchwald’s (2009)14 meta-analysis where remission of diabetes after RYGB or BPD surgery is close to double that observed after LAGB (Table 1-2). Furthermore, diabetes remission occurs rapidly after RYGB surgery, typically within days to weeks of surgery.12 Such rapid remission is not observed after restrictive bariatric procedures (LAGB, VBG), which are thought to improve glucose homeostasis mainly by inducing weight loss over an extended period of time.436, 440, 446 In a study of 1160 individuals undergoing RYGB surgery, one third of those with diabetes were normoglycaemic without diabetic medication within 3 days of surgery.447 Similarly, a study by Wickremesekera et al. (2005) observed rapid remission of diabetes after RYGB surgery which was associated with a dramatic improvement in insulin sensitivity.448 Increasing evidence suggests that manipulation of the gastrointestinal tract can cause weight independent anti-diabetic effects. Initial development of experimental bariatric surgeries like the duodenal-jejunal bypass (DJB) by Rubino and Marescaux (2004) provided compelling evidence that the RYGB surgery causes remission of diabetes by mechanisms other than weight loss.449
Non-obese rats with type 2 diabetes that underwent DJB showed marked and durable improvement in glucose homeostasis regardless of caloric intake or weight loss. Other studies that used the same animal model reported similar findings.\textsuperscript{450-452}

Subsequent observational studies comparing RYGB surgery with other bariatric surgeries or weight loss treatment provided further evidence for weight independent mechanisms of diabetic remission. LaFerrère et al. (2008) showed that despite equivalent weight loss, individuals who had RYGB surgery had a greater improvement in glucose homeostasis than those who achieved weight loss by dieting.\textsuperscript{453} Similarly, a prospective study following individuals who had equivalent weight loss after RYGB or LAGB surgery demonstrated significantly high rates of diabetic remission in the RYGB group (72\% versus 17\%).\textsuperscript{454} Consequently, the weight independent effects of bariatric surgery have led to the concept of “metabolic” surgery as a possible treatment for type 2 diabetes in overweight individuals.\textsuperscript{455}

1.10.4 Mechanisms behind remission of diabetes after bariatric surgery

Two major hypotheses have been proposed to explain the early effects of RYGB surgery on regulation of glucose homeostasis.\textsuperscript{456, 457} The foregut hypothesis states that rapid delivery of nutrients to the distal small intestine stimulates L-cells, which secrete hormones (also called incretins) that augment insulin secretion and/or action resulting in improved homeostasis. Conversely, the hindgut hypothesis states that excluding parts of the small intestine reduces or suppresses release of anti-incretin factors that decrease insulin secretion and/or action thus improving glucose homeostasis.

Although no evidence suggests the foregut and hindgut hypothesis are mutually exclusive, several studies have reported altered incretin secretion after gastric bypass surgery. These studies have reported levels of peptide YY (PYY), glucagon like peptide-1 (GLP-1), gastric inhibitory peptide (GIP) and ghrelin to be altered after RYGB surgery.\textsuperscript{458-463}

RYGB surgery has been observed to induce early changes in incretin levels before any substantial weight loss.\textsuperscript{463} However, whether these incretins are involved in the
rapid remission of diabetes after RYGB surgery remains controversial. A study from our own group has shown there were no significant changes in GLP-1, GIP or PYY levels within days of RYGB surgery despite normalization of fasting glucose levels (manuscript submitted).

The rapid exposure of the foregut to food has also led to formation of two other hypotheses. The ability of incretins to signal through neuronal networks has led to the suggestion that a gut-brain-liver axis may have a role in remission of diabetes after RYGB surgery. Cholecystokinin (CCK) is a peptide hormone released by the gut in response to nutrients and has been shown to lower glucose production through a neural network.464 Similarly, central nervous system GLP-1 receptors have been shown to have a role in enteric sensing of glucose ingestion that modulates peripheral glucose metabolism, which is impaired in diabetes.465

The role of intestinal nutrient sensing has also been directly implicated in the anti-diabetic effect of RYGB surgery. Troy et al. (2008) suggested that increased intestinal gluconeogenesis after RYGB surgery elevates the portal vein glucose concentration. This is thought to activate the hepatic glucose portal sensing pathway, which decreases hepatic glucose output and improves glucose homeostasis.466 However, a study by Hayes et al. (2011) showed that there were no significant differences between portal and peripheral glucose concentrations after the first six days of RYGB surgery.467 This suggests intestinal gluconeogenesis is probably not involved in the rapid remission of diabetes after RYGB surgery.

1.10.5 Tissue specific changes in insulin action after RYGB surgery

Regardless of the mechanism behind the anti-diabetic effects of RYGB surgery manipulation of the gastrointestinal tract can significantly improve insulin sensitivity and β-cell function.468, 469 Improved insulin secretion and restoration of the first phase insulin response has been associated with normalization of glucose homeostasis after gastric bypass surgery.470-472 Furthermore, rapid resolution of insulin resistance is associated with a return to normoglycemia after RYGB surgery (Figure 1.10-2).448
Although others have reported similar findings, the mechanisms behind the improvement in insulin resistance after RYGB surgery remain unclear. Several studies have suggested that both significant weight loss and decreased energy intake are responsible for the improvement in insulin sensitivity. However, other studies have shown that even though strict dieting can improve insulin resistance, the magnitude of improvement is higher in individuals who have undergone RYGB. Nevertheless, resolution of insulin resistance occurs rapidly after gastric surgery and appears to be tissue specific.

Lima et al. (2010) measured both peripheral (euglycemic clamp) and hepatic (HOMA-IR) insulin resistance in individuals undergoing RYGB surgery. One month after surgery, hepatic insulin resistance was resolved, whereas peripheral insulin resistance remained unchanged. In the same year, Campos et al. (2010) reported that rapid normalization of glucose homeostasis was independent of peripheral insulin resistance, which only resolved when substantial weight loss occurred. Similarly, a study by Foo et al. (2011) showed that normalization of glucose homeostasis within six days of RYGB surgery was associated with resolution of hepatic insulin resistance (HOMA-IR), while peripheral insulin resistance (IVITT) increased. Taken together, these data highlight the importance of the liver in remission of type 2 diabetes after RYGB surgery.
1.11 Hypothesis and Aims

Recent advances have increased our understanding of insulin resistance and type 2 diabetes. Nonetheless, effective treatments that do not involve surgical manipulation remain elusive. Bariatric surgery, and especially RYGB surgery, has been shown to cause rapid remission of type 2 diabetes and insulin resistance. This provides the scientific community with an unprecedented human model that can be used to investigate the pathogenesis of type 2 diabetes.

We hypothesize that the liver is critical to remission of diabetes after RYGB surgery and has a central role in the pathogenesis of type 2 diabetes. There are multiple lines of evidence supporting this hypothesis:

- The liver has significant contributions to metabolic homeostasis via regulation of glucose and lipid production.
- The liver regulates insulin action in other tissues by modulating the peripheral insulin concentration via hepatic insulin clearance.
- Transgenic animal studies have demonstrated that the liver may be more critical than muscle and fat tissues in regulating glucose homeostasis.
- Primary hepatic insulin resistance may cause whole body insulin resistance that leads to type 2 diabetes.
- Resolution of hepatic insulin resistance is thought to be critical to the remission of type 2 diabetes after RYGB surgery.
- Resolution of liver steatosis has been associated with improvement in regulation of glucose homeostasis.

Consequently, this thesis explores the role that hepatic molecular insulin resistance has in the pathogenesis of type 2 diabetes.
Although critical to insulin action in the periphery, hepatic insulin clearance has been largely overlooked as a mechanism that may be involved in type 2 diabetes. Likewise, even though the IR and its isoforms have been known for 30 years, their involvement in type 2 diabetes is still controversial. The broad aim of this thesis is to use the RYGB as a human model to further investigate previously implicated as well as identify novel molecular processes involved in the pathogenesis of type 2 diabetes. This aim will be achieved by answering the following questions.

- Does gene and protein expression of molecules that modulate insulin signalling (ENPP1) and hepatic insulin clearance (CEACAM-1 and IDE) change following the improvement in insulin resistance and remission of diabetes achieved by RYGB?
- Do the insulin receptor isoforms A and B have a role in the pathology of type 2 diabetes?
- What global changes occur in hepatic gene expression in individuals having RYGB who experience normalization of insulin sensitivity and remission of type 2 diabetes.
CHAPTER TWO: Roux-en Y gastric bypass surgery can be used to study type 2 diabetes
2.1 Introduction: using models to study type 2 diabetes

The use of model organisms to study disease is essential to molecular biology. The most commonly employed model organisms in diabetes are the mouse and rat. Although larger animals such as dogs and pigs have also been used, the move to transgenic animal models has seen the rise of the rodent as the model of choice for dissecting the insulin signalling pathway. With these models, we can control for environmental factors allowing us to characterise single genes.

To advance our understanding of type 2 diabetes pathogenesis even further, we need models that represent the environmental and genetic variability seen in a diverse human population. Research in humans has been limited to clinical and molecular experiments using easily accessible samples such as blood and muscle or fat tissues. However, bariatric surgery not only provides an excellent human model for the study of type 2 diabetes, but also allows for access to liver tissue during surgery. Further, some individuals may need added surgery for related or unrelated complications (e.g. cholecystectomy, incisional hernia, ring removal) and as a result will be available for repeat biopsy of tissues. For the first time it becomes possible to examine tissue in individuals with diabetes and the same individual following remission of diabetes by RYGB. This creates a powerful tool for exploring the pathogenesis of type 2 diabetes and provides an opportunity exploited in this thesis.

The studies reported in this PhD thesis describe experiments performed on a subgroup of patients undergoing RYGB at Wakefield Hospital in recent years. All patients undergoing RYGB surgery since 2000 for severe obesity at Wakefield Hospital were considered for data and specimen collection. At surgery, various blood and tissue samples were collected including a liver biopsy. This chapter characterises the study group used in this thesis to explore the liver molecular mechanisms behind insulin resistance and type 2 diabetes.
2.1.1 Experimental strategy

- Individuals selected for the study were demographically profiled and metabolically characterised using a range of clinical tests that included measurement of fasting plasma glucose, fasting plasma insulin and HbA1c.

- Insulin resistance was estimated using the HOMA-IR algorithm, whereas glucose tolerance and type 2 diabetes was identified using a oral glucose tolerance test.

- Individuals were then categorised into study groups based on their metabolic characteristics that included: normal glucose tolerance, impaired glucose tolerance and type 2 diabetes.

- The metabolic characteristics of the subsets of individuals who had a second liver biopsy after RYGB surgery are presented alongside the main study groups for comparisons.
2.2 Research Design and Methods

All patients on whom data and tissue is available before and/or after RYGB surgery for obesity (>35kg/m²) gave informed consent under ethical approval from the Central Regional Ethics Committee (Wellington, New Zealand). All surgery was performed by the same surgeon each time (Prof. Richard Stubbs) and all procedures were approved by the Central Regional Ethics Committee.

2.2.1 Pre operative data collection and type 2 diabetes diagnosis

Clinical data was collected 2-6 weeks prior to surgery which included, but was not limited to: weight, height, body mass index, glycated haemoglobin (HbA1c), fasting plasma glucose, and fasting plasma insulin.

Diagnosis of type 2 diabetes was established in two ways. First, an individual was diagnosed with type 2 diabetes if there was prior documentation of diagnosis and/or if the individual was receiving treatment for type 2 diabetes. Alternatively, diabetes was established by an oral glucose tolerance test (OGTT) which was routinely performed in all patients without a known history of diabetes. Previously unrecognised diabetes was established by either:

- Fasting glucose $\geq 7.0$ mmol/L and/or a
- OGTT 2 hour glucose $\geq 11.1$ mmol/L.

Individuals who had a fasting glucose $< 7.00$ mmol/l, but an OGTT 2 hour glucose $\geq 7.8 \leq 11.1$ mmol/l were diagnosed with impaired glucose tolerance. Individuals with type 2 diabetes were further classified as previously unrecognized, diet controlled, requiring oral hypoglycaemic drugs or insulin taking.
2.2.2 Subject selection and grouping

For the studies described in this thesis a subset of 55 patients (from a database of >1000) was selected based on availability of fresh frozen liver tissue taken at the time of RYGB surgery. This study cohort included individuals who had: normal glucose tolerance (NGT group), impaired glucose tolerance (IGT group), and type 2 diabetes (T2DM group).

Out of those 55, 16 individuals had repeat liver biopsy at the time of subsequent operations. These individuals were further classified into two re-operation groups as follows: individuals that had a second liver biopsy and had normal glucose tolerance (sNGT group) and individuals who had a second liver biopsy and had type 2 diabetes (sT2DM group). Table 2-1 shows relevant anthropometric data for the five study groups around the time of RYGB surgery

Table 2-1. Anthropometric data of 55 obese individuals classified by study group.

<table>
<thead>
<tr>
<th>Variables</th>
<th>NGT (n=19)</th>
<th>sNGT (n=8)</th>
<th>IGT (n=9)</th>
<th>T2DM (n=27)</th>
<th>sT2DM (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>44 ± 8</td>
<td>43 ± 9</td>
<td>47 ± 10</td>
<td>54 ± 7</td>
<td>50 ± 7</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>46 ± 6</td>
<td>44 ± 5</td>
<td>49 ± 4</td>
<td>49 ± 10</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (%)</td>
<td>16 (84)</td>
<td>6 (75)</td>
<td>6 (66)</td>
<td>15 (55)</td>
<td>5 (63)</td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previously unrecognized (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7 (27)</td>
<td>3 (37.5)</td>
</tr>
<tr>
<td>Diet Controlled (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2 (8)</td>
<td>1 (12.5)</td>
</tr>
<tr>
<td>Oral hypoglycaemics (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14 (50)</td>
<td>4 (50)</td>
</tr>
<tr>
<td>Insulin taking (%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4 (15)</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are presented as mean± SD

All individuals were morbidly obese at the time of surgery (BMI>40 kg/m²) and there were no statistically significant differences in BMI between any of the groups, while females tended to be overrepresented particularly in the NGT and sNGT group.
2.2.3 Tissue sampling and follow up data collection

At the time of surgery a variety of tissue samples were collected including a Tru-Cut® Soft Tissue Biopsy Needle (Cardinal Health) liver biopsy. Follow up data was taken at three and 12 months after RYGB surgery. At each assessment, data collection included, weight, body mass index, HbA1c, fasting plasma glucose and fasting plasma insulin.

A second liver biopsy was taken from a limited number of patients (~16 months after RYGB surgery) who returned for further surgery which allowed access to the liver. Almost all of the subsequent surgeries were incisional hernia repairs, except two, which were ring removal procedures. For those individuals who had a second liver biopsy, clinical measurements were taken within 2 months of subsequent surgery.

Remission of type 2 diabetes was defined according to the criteria of Buse et al. (2009), which is as follows:

- Partial remission was documented if, one year after surgery, the HbA1c% was <6.5%, fasting plasma glucose was 5.6-6.9 mmol/l and there was no ongoing treatment.

- Complete remission was documented if, one year after surgery, the HbA1c% was <6.5%, fasting plasma glucose was <5.6 mmol/l and there was no ongoing treatment.

2.2.4 Biochemical testing

All patients undertook a 12 hour fast prior to blood collection for biochemical tests. Fasting was either self administered or administered during the hospital stay. Clinical biochemistry testing was conducted by Aotearoa Pathology (Wellington, New Zealand) while testing for insulin was undertaken at Canterbury Health Laboratories (Christchurch, New Zealand), both of which are accredited by International Accreditation New Zealand (IANZ).

* The particular gastric bypass procedure performed at Wakefield Hospital is the Fobi Pouch, which includes a silastic ring to define the size of the gastric outlet. In approximately 2% of individuals this is subsequently removed to improve quality of eating.
Glucose was assayed on the Roche Modular P800 Chemistry Analyzer using standard Roche reagent, whereas HbA1c levels were assayed on the Variant Turbo Ion Exchange HPLC platform. Insulin was assayed using the Roche Elecsys 2010 automated analyzer and standard Roche reagent. Below is a brief description of the specimen collection and method principal for each assay.

### 2.2.4.1 Glucose assay and method principle

Blood was collected in a BD Vacutainer® tube containing K₂C₂O₄ (anticoagulant) and NaF (antiglycolytic agent). Glucose concentrations were assayed using an enzymatic method with hexokinase⁴⁷⁹ (Figure 2.2-1). In this method, hexokinase catalyzes the phosphorylation of glucose to glucose-6-phosphate. Glucose-6-phosphate dehydrogenase then oxidizes glucose-6-phosphate in the presence of NADP to gluconate-6-phosphate. The rate of NADPH formation is measured photometrically and is directly proportional to the concentration of glucose in the sample.

![Figure 2.2-1. Glucose method principle. An enzymatic method that uses the rate of NADPH formation to indirectly measure the concentration of glucose in a sample.](image)

### 2.2.4.2 HbA1c assay

Blood was collected in a BD Vacutainer® tube containing K₂EDTA (anticoagulant). HbA1c was assayed on the Variant Turbo Ion Exchange High Performance Liquid Chromatography (HPLC) platform. Briefly, HbA1c is glycated haemoglobin in which glucose is bound to the N-terminal valine of the haemoglobin β-chain. This changes its conformation resulting in a molecule with an extra negative charge. Using ion exchange HPLC, Hb species can be separated based on the net charge differences between HbA1c and different haemoglobins, thus allowing for its measurement.
2.2.4.3 Insulin assay and method principle

Blood was collected in a BD Vacutainer® tube containing K₃EDTA (anticoagulant). Plasma was separated from the red blood cells by centrifuging the tubes at 1300 RCF for 10 minutes and stored at -80°C before being sent frozen to Canterbury Health Laboratories for insulin analysis. Prior to analysis, plasma was treated with an equal volume of 25% PEG 6000 to precipitate immunoglobulins that may interfere with the assay.

Following this, the insulin concentration was measured using a two-site non-competitive sandwich assay that uses two monoclonal antibodies. Briefly, in the first step the sample is incubated for nine minutes with a reagent containing biotinylated insulin antibody and a ruthenium labelled insulin antibody, both of which bind to the free insulin in the sample. Streptavidin-coated paramagnetic microparticles are then added, which bind to the biotin labelled insulin antibody. The reaction mixture is then transported to the measuring cell where the immune complexes are magnetically trapped on the working electrode and the unbound reagent and sample are washed away. An electrical charge is then applied which excites the bound ruthenium and stimulates an electrochemiluminescent reaction that produces light. The amount of light produced is directly proportional to the amount of insulin in the sample.

2.2.5 Estimation of insulin resistance

Homeostasis model assessment (HOMA) was used to estimate insulin resistance using the following formula;

\[
HOMA-IR = \frac{\text{fasting plasma insulin (mU/l)} \times \text{fasting plasma glucose (mmol/l)}}{22.5^{.88}}
\]

HOMA-IR less than 2.2 has been shown to be representative of a metabolically normal, insulin sensitive population.09
2.2.6 Statistical analysis

Normal distribution of all groups to be compared was tested with D’Agostino and Pearson omnibus normality test. Non-normally distributed data was log transformed to comply with parametric test assumptions. Pre-post comparisons were carried out using a paired t-test. One way analysis of variance (ANOVA) with Bonferroni correction was used to test for significant differences between means. General linear model (GLM) with Tukey pairwise comparison was used to test for significant differences in means between multiple factors. An alpha level of 0.05 was set as the significance threshold. All analysis was performed using Minitab15. Graphical visualization was performed using GraphPad Prism 5.
2.3 Results

In order to achieve the aims set out in this thesis, the metabolic status of the study cohort used was characterised by investigating several clinical parameters before and after RYGB surgery. These parameters included: weight loss (via change in BMI), insulin resistance (fasting insulin and HOMA-IR), and glucose homeostasis (fasting glucose and HbA1c).

2.3.1 Weight loss after bariatric surgery

Individuals were followed up with measurements recorded at three and 12 months after surgery. Out of the 55 individuals included in the study, three had incomplete follow up BMI data. BMI measurements were logarithmically transformed prior to statistical analysis using GLM, while Figure 2.3-1 presents the mean BMI values for the five groups at each of the time points measured. The NGT, IGT and T2DM groups all had a significant decrease in BMI within 3 months of surgery, which decreased further at the one year time point.

![Figure 2.3-1. Mean BMI ± SD after RYGB surgery in all individuals and the subset that had a second liver biopsy. *The change in BMI observed 3 and 12 months after RYGB surgery in the NGT, IGT and T2DM groups was significantly different from pre-RYGB values (GLM).](image-url)
The subset of individuals who had a second liver biopsy had comparable weight loss to the rest of the study group. Notably, there was no statistically significant difference in weight loss between the IGT, NGT or T2DM group (p=0.131) and the sNGT and sT2DM group (p=0.180) at any time point measured.

2.3.2 Improvements in insulin resistance after RYGB surgery

Biochemical parameters were measured at 3 and 12 months after RYGB surgery. Out of the 55 individuals included in the study, 12 had incomplete follow up biochemical data. Insulin resistance was estimated by calculating HOMA-IR at each of the time points. Fasting plasma insulin concentrations and HOMA-IR were logarithmically transformed prior to statistical analysis using GLM and Tukey’s pairwise comparison. Figure 2.3-2 presents the untransformed means for fasting insulin and HOMA-IR for the five groups at each of the time points measured.

Fasting insulin concentrations fell significantly following RYGB surgery in the NGT, IGT and T2DM group (Figure 2.3-2A and C), which was statistically significant (P<0.001). The magnitude of reduction in insulin concentrations was similar in all groups. As expected, the T2DM group had a higher mean insulin concentration than the NGT group (p=0.02), although this difference normalised after the operation as there were no statistically significant differences in insulin concentrations between any of the groups after RYGB surgery. The same fall in fasting insulin was seen in the subgroup of those having a second liver biopsy (sNGT and sT2DM group).

Insulin resistance improved dramatically after RYGB as evidenced by the statistically significant (p<0.001) decreases in HOMA-IR values in the NGT, IGT and T2DM groups (Figure 2.3-2C). Before the surgery, the NGT, IGT and T2DM groups all had mean HOMA-IR values above 2.4 indicating varying degrees of insulin resistance. As expected, the T2DM had the highest mean HOMA-IR value, which was significantly higher that the mean HOMA-IR of the NGT group (p<0.001). Within 3 months of surgery, the values decreased to 1.46 (± 0.98 SD), 2.3 (± 0.94 SD), and 2.44 (± 1.71 SD) for the NGT, IGT and T2DM group respectively.
By the one year time point all three groups had a statistically significant (p<0.001) decrease in HOMA-IR values. The subset of individuals who underwent a second liver biopsy had similar HOMA-IR profiles to the main study groups after RYGB surgery (Figure 2.3-2B and D). Furthermore there were no statistically significant differences in HOMA-IR between any of the groups 3 months after surgery.
2.3.3 Remission of diabetes after RYGB surgery

Fasting plasma glucose concentrations were logarithmically transformed prior to analysis with GLM and Tukey’s pairwise comparison. Figure 2.3-3 shows the untransformed means of glucose and HbA1c three and 12 months after RYGB surgery. The T2DM group had the highest mean glucose concentrations, which normalised within 3 months of RYGB surgery, and remained below the diagnostic level for type 2 diabetes (fasting plasma glucose of $\geq 7$ mmol/l) at 12 months. Individuals with normal or impaired glucose tolerance; however, had little to no changes in mean glucose concentrations (Figure 2.3-3A and B).

![Graph of mean fasting plasma glucose and HbA1c concentrations](image)

Figure 2.3-3. Change in mean fasting plasma glucose concentrations and HbA1c ± SD in the NGT, IGT and T2DM groups (A and C) and in the sNGT and sT2DM groups (B and D) up to one year after RYGB surgery. *Difference in glucose concentration and HbA1c between the NGT and T2DM was statistically significant (GLM). **Changes in fasting glucose and HbA1c from Pre-RYGB values were statistically significant 3 and 12 months after RYGB only in the T2DM group (GLM).
As expected, HbA1c levels followed a similar pattern to the changes in fasting plasma glucose after RYGB surgery. The T2DM group had the highest mean HbA1c levels, which were diagnostic of type 2 diabetes. Following RYGB surgery, only the T2DM group had a decrease in mean HbA1c levels whereas the NGT and IGT groups had no appreciable changes. Within 3 months of surgery, there were no differences in mean HbA1c levels between any of the groups. Furthermore, 12 months after surgery, the T2DM group had a mean HbA1c level of 5.85 (± 0.71 SD), which was below the diagnostic criteria for type 2 diabetes (HbA1c ≥6.5%). The sNGT and sT2DM groups had similar mean glucose and HbA1c profiles to the main study groups after RYGB surgery (Figure 2.3-3 B and D).

Remission of diabetes was based on fasting glucose concentrations and HbA1c levels one year after RYGB surgery. Out of the 27 individuals who had type 2 diabetes, 18 were shown to have complete remission, six had partial remission after surgery, two had no remission and one was unknown (due to a missing 12 month HbA1c value).

2.3.4 Metabolic status during liver biopsy

Liver tissue from each individual in the five study groups was used throughout this thesis to investigate the molecular processes affected by insulin resistance or type 2 diabetes. Table 2-2 shows metabolic characteristics of the NGT, IGT and T2DM groups around the time of initial liver biopsy (pre RYGB surgery). At the time of initial liver biopsy, the T2DM group had significantly (p<0.001, ANOVA) higher HbA1c and fasting glucose levels than both the NGT and IGT groups, while all groups had abnormally high plasma insulin concentrations and some level of insulin resistance. The NGT group had the lowest insulin resistance while the T2DM group had the highest as inferred from HOMA-IR. The plasma insulin concentrations were only significantly different between the T2DM and the NGT group (p<0.001, ANOVA) as were the HOMA-IR values (p<0.001, ANOVA).
Table 2-2. Metabolic status at the time of initial liver biopsy

<table>
<thead>
<tr>
<th>Variables</th>
<th>NGT group (n=19)</th>
<th>IGT group (n=9)</th>
<th>T2DM group (n=27)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>46 ± 6.0</td>
<td>49 ± 3</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>5.5 ± 0.4</td>
<td>5.6 ± 0.5</td>
<td>7.6 ± 1.1*</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>3.70 ± 3.6</td>
<td>6.07 ± 3.1</td>
<td>10.78 ± 8.6*</td>
</tr>
<tr>
<td>Fasting plasma insulin (pmol/L)</td>
<td>109 ± 96</td>
<td>164 ± 80</td>
<td>196 ± 115†</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/L)</td>
<td>5.1 ± 0.6</td>
<td>5.7 ± 0.6</td>
<td>8.1 ± 2.3*</td>
</tr>
</tbody>
</table>

NGT vs T2DM data are significantly different; *p<0.001, †p<0.05 (ANOVA). Values are presented as mean± SD.

Individuals in the sNGT and sT2DM groups all had a second liver biopsy at subsequent operations. Metabolic characteristic for the sNGT and sT2DM groups at the time of initial and second liver biopsy are listed in Table 2-3 for comparison. Out of the 16 individuals who had a second liver biopsy, two had incomplete follow up data. Both the sNGT and sT2DM groups had significant decreases in BMI by the time of the second biopsy. There were no statistically significant differences in BMI between the two groups at either operation or in the amount of weight lost.

Table 2-3. Metabolic status of individuals at the time of initial and subsequent liver biopsy.

<table>
<thead>
<tr>
<th>Variables</th>
<th>sNGT group (n=8)</th>
<th>sT2DM group (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-RYGB</td>
<td>Post-RYGB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>44 ± 5</td>
<td>28 ± 4*</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>5.4 ± 0.2</td>
<td>5.0 ± 0.7</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>2.5 ± 0.45</td>
<td>0.78 ± 0.34†</td>
</tr>
<tr>
<td>FPI (pmol/L)</td>
<td>80 ± 16</td>
<td>27 ± 13*</td>
</tr>
<tr>
<td>FPG (mmol/L)</td>
<td>4.9 ± 0.4</td>
<td>4.7 ± 0.4</td>
</tr>
</tbody>
</table>

Pre-RYGB vs post-RYGB data are significantly different *p<0.001, †p<0.05 (Paired t-test). Values are presented as mean± SD.
For the sT2DM group, there was a significant (p < 0.05, Paired t-test) decrease in mean fasting plasma glucose and HbA1c levels, which was representative of complete remission of type 2 diabetes in all except one individual who had a missing 12 month HbA1c value and thus could not be categorized.

Hepatic insulin sensitivity (as measured by HOMA-IR) improved dramatically in both the sNGT and sT2DM group, even though the sNGT group were already relatively insulin sensitive before RYGB surgery. Similarly, fasting plasma insulin concentrations fell significantly in both groups. Nonetheless, the sT2DM had the greatest improvement in insulin sensitivity which was associated with the normalisation of insulin resistance in those individuals.


2.4 Discussion

Bariatric surgery has provided the scientific community with an unprecedented human model of type 2 diabetes. RYGB surgery in particular induces drastic improvements in glycaemic control in morbidly obese individuals.14,430,439 This chapter characterises the metabolic status of individuals who contributed tissue for investigation of liver molecular mechanisms involved in insulin resistance and type 2 diabetes.

All individuals included in this study were morbidly obese (BMI>40 kg/m²) and as expected all had a great and continuous decrease in BMI up to one year after RYGB surgery (Figure 2.3-1). Although weight loss undoubtedly has a role in the improvements seen in metabolic homeostasis, RYGB surgery has been shown to induce normoglycemia without significant weight loss.448, 454, 475, 480 Further, while liver insulin resistance as measured by HOMA-IR decreases rapidly post RYGB surgery, whole body insulin resistance only decreases with weight loss and may remain for up to six months after surgery.448, 476, 477, 480, 481 This strongly suggests the liver is central to remission of type 2 diabetes after RYGB surgery.

We grouped the individuals included in the study according to their metabolic characteristics. As expected mean fasting plasma glucose and HbA1c levels were normal in the NGT group (Figure 2.3-3A and C), and slightly raised in the IGT group. However there were no statistically significant differences between the NGT and IGT group in any of the parameters measured at RYGB surgery. In addition, the IGT group had a smaller sample size in comparisons to the NGT and T2DM groups and as a result we excluded the IGT group from the studies described in this thesis.

Unlike the NGT group, the T2DM group had significantly raised mean fasting plasma glucose and HbA1c values representative of individuals with type 2 diabetes (Figure 2.3-3A and C). Although insulin resistance was present in varying severity in all groups, the T2DM group were the most insulin resistant as they had the highest mean fasting plasma insulin concentration (Figure 2.3-2A) and HOMA-IR values (Figure 2.3-2C).
The mechanisms governing improvement in metabolic homeostasis after RYGB surgery are unclear. Nonetheless, improvements in insulin sensitivity are critical to normalisation of glucose homeostasis in individuals with type 2 diabetes. In this study, RYGB surgery induced an increase in insulin sensitivity regardless of disease presence. Interestingly, the NGT and T2DM groups both had a significant decrease in fasting plasma insulin concentrations and HOMA-IR values within a year of surgery (Figure 2.3-2A and C). Resolution of insulin resistance in the T2DM group coincided with remission of diabetes in 88% of individuals, which is similar to remission rates previously reported. Although two individuals did not have remission of diabetes, they did have an improvement in insulin resistance as judged by HOMA-IR, which was < 2.4 for both individuals.

One of the novel characteristics of this thesis is the use of liver tissue biopsied from the same individual before and after significant improvement in insulin sensitivity and/or remission of type 2 diabetes. Figures 2.3-1, 2.3-2 and 2.3-3 show that both the sNGT and sT2DM group had similar changes in metabolic characteristics as the groups they stemmed from (NGT and T2DM group). Even though most of the sNGT group were insulin sensitive at RYGB surgery, they still had a significant increase in insulin sensitivity at second liver biopsy. The sT2DM group had resolution of insulin resistance at second liver biopsy as the mean HOMA-IR decreased to from 10.3 (± 6.2 SD) to 1.4 (±0.8 SD), which was well within the insulin sensitive range (HOMA IR <2.4). Resolution of insulin resistance in the sT2DM group coincided with the remission of diabetes as shown by normalisation of mean fasting plasma glucose concentrations and HbA1c levels (Table 2-3). Importantly, there were no differences in BMI between the sNGT and sT2DM groups at any one time point measured (Figure 2.3-1) nor at the time the time of the second liver biopsy (Table 2-3). This removed weight loss as a possible confounding factor.

Collectively, the data presented here shows that individuals used in this study suitably model insulin resistance and type 2 diabetes. The individuals grouped in the NGT and sNGT groups had normal glucose homeostasis and were relatively insulin sensitive. Individuals grouped in the T2DM and sT2DM groups had deranged glycaemic control and were severely insulin resistant. Further, RYGB surgery induced significant improvements in insulin sensitivity in all individuals which coincided with remission
of diabetes in those individuals that had the disease. We used these study groups to answer two key questions asked throughout the thesis. First, what are the molecular differences in liver insulin signalling between individuals with or without type 2 diabetes? Second, how do pathways involved in insulin action in the liver change after resolution of insulin resistance and what role do they have in remission of type 2 diabetes. The next two chapters use the RYGB model to explore several key molecules involved in insulin action and whether these molecules have a significant role in the pathogenesis of insulin resistance and type 2 diabetes.
CHAPTER THREE: Investigation of molecules involved in the modulation of insulin signalling strength and hepatic insulin clearance
3.1 Introduction

Insulin action is dependant on the circulating insulin concentration and the consequent activation of the insulin receptor tyrosine kinase. Investigating the mechanisms that govern insulin action is critical to our understanding of insulin resistance and type 2 diabetes. This chapter presents data on two key processes involved in insulin action, namely hepatic insulin clearance and negative regulation of IR activity.

Negative regulation of IR activity through various mechanism has previously been implicated in the pathogenesis of insulin resistance.\textsuperscript{158} One of these mechanism is the inhibition of insulin binding to the IR α-subunit by ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1 also referred to as plasma cell membrane glycoprotein 1 or PC-1).\textsuperscript{166} Increased ENPP1 protein levels have been found in skeletal muscle,\textsuperscript{167} adipose tissue\textsuperscript{168} and skin fibroblasts\textsuperscript{169, 170} from insulin-resistant individuals. Overexpressing ENPP1 \textit{in vitro}\textsuperscript{170} and \textit{in vivo}\textsuperscript{172} led to impaired insulin signalling and action, while selectively suppressing the levels of ENPP1 in the liver of mice improved insulin sensitivity.\textsuperscript{173} Moreover, there is compelling evidence suggesting a role for ENPP1 in genetic forms of insulin resistance. Genotype-phenotype association studies have identified a gain of function mutation (K121Q), which increases the inhibitory function of ENPP1 and may have a role in insulin resistance and type 2 diabetes.\textsuperscript{174, 175}

Hepatic insulin clearance mediates insulin action by regulating the systemic insulin concentration and has been found to be diminished in obesity and type 2 diabetes.\textsuperscript{57-60} Although the pathological importance of this process is unclear, a recent study by Bojsen-Møller et al. (2013)\textsuperscript{482} has shown that hepatic insulin clearance increases as early as one week after RYGB surgery. The liver regulates insulin clearance by modulating the rate at which insulin is internalized and degraded. Two key molecules involved in this process are carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM-1) and insulin degrading enzyme (IDE). CEACAM-1 is involved in mediating the internalization of the insulin-insulin receptor complex.\textsuperscript{64-66} Transgenic mice with aberrant CEACAM-1 expression or action have impaired insulin clearance, secondary insulin resistance, abnormal glucose tolerance and visceral adiposity.\textsuperscript{64, 65, 70} On the other hand, IDE is responsible for the degradation of insulin\textsuperscript{63} and has
previously been implicated in the pathogenesis of type 2 diabetes.\textsuperscript{72-74} Furthermore, inhibitors of IDE have previously been proposed as a potential anti-diabetic therapy.\textsuperscript{71}

Despite the evident roles of ENPP1, CEACAM-1 and IDE in insulin resistance, they are yet to be extensively characterised in liver tissue of human individuals with and without type 2 diabetes. Consequently, this chapter presents the gene expression and protein abundance of the three aforementioned molecules in individuals with and without type 2 diabetes before and after RYGB surgery.

3.1.1 Experimental strategy

- Protein abundance was assayed in the NGT, T2DM, sNGT and sT2DM group using E-PAGE™ 48-well protein electrophoresis and western blotting.

- Gene expression was assayed in the NGT, T2DM, sNGT and sT2DM group using RT-qPCR with TaqMan Gene Expression assays and the Relative Quantification method.
3.2 Research Design and Methods

Metabolic characteristics of individuals from whom liver tissue was available are presented in Chapter II. Liver gene expression and protein abundance of ENPP1, CEACAM-1 and IDE was assayed in individuals with type 2 diabetes (T2DM group) or normal glucose tolerance (NGT group). In addition, gene expression and protein abundance was also assayed after RYGB surgery (and remission of diabetes) in individuals with normal glucose tolerance (sNGT group) or type 2 diabetes (sT2DM group). In this way we expected to learn something of the role of these genes in the development of type 2 diabetes.

3.2.1 RNA extraction

Total RNA was phenol chloroform extracted by a method based on a technique developed by Chomczynski et al. (1987). Briefly, 2-3mg of frozen liver tissue was pulverized under liquid nitrogen after which 800\(\mu\)l of TRIzol® (Life Technologies), a monophasic solution containing phenol and guanidinium thiocyanat, was added to the resultant powder. The mixture was then homogenised with an Omni Tissue Homogenizer (Omni International) and 200 \(\mu\)l of chloroform was added, vigorously mixed then centrifuged at 12,000g for 15 minutes at 4-8ºC. Following centrifugation the mixture separates into a red phenol-chloroform phase, an interphase and a colourless upper aqueous phase containing RNA.

To precipitate the RNA, the aqueous phase was transferred to an RNAase/DNAase free Eppendorf tube containing 500\(\mu\)l of isopropyl alcohol and 1.3 \(\mu\)l (conc) GlycoBlue (Life Technologies). GlycoBlue is a blue dye covalently linked to glycogen and was used as a co-precipitant to increase RNA pellet mass and aid in pellet visualization. Following 10 minute incubation, samples were centrifuged at 12,000 g for 10 minutes at 4-8ºC. The resultant RNA pellet was washed with 75% ethanol and air dried for 10 minutes. Depending on pellet mass, 20-30\(\mu\)l of DEPC-treated water was used to re-suspend the RNA pellet after which it was incubated at 55ºC for 10 minutes. RNA was stored at -80 ºC prior to quality control and use in first strand cDNA synthesis.
3.2.2 RNA quality control

Quality and quantity of RNA was analyzed on the 2100 Agilent Bioanalyzer, using the Agilent RNA 600 Nano kit and BioSizing software (Agilent Technologies). The Agilent 2100 Bioanalyzer uses microfluidic capillary electrophoresis to separate RNA samples according to their molecular weight and then detects them using laser-induced fluorescence detection. The Biosizing software produces an electropherogram where the amount of fluorescence correlates with the amount of RNA. An RNA integrity number (RIN) was calculated by an algorithm which calculates the RNA integrity from the electrophoretic data using a trained artificial neural network. A RIN number was computed for each RNA profile and classified it into one of 10 predefined categories of integrity. A RIN of 1 represented a completely degraded sample, whereas a RIN of 10 represented an intact RNA sample. A RIN of 5.5 or higher is appropriate for RT-qPCR experiments. Table 3-1 lists mean RNA integrity numbers for all the groups that were compared. All RIN numbers were above the 5.5 threshold for RT-qPCR and as such passed quality control.

Table 3-1. Mean RNA integrity number for all groups being compared.

<table>
<thead>
<tr>
<th>Study Group</th>
<th>Mean RIN (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGT group</td>
<td>7.1 (1.0)</td>
</tr>
<tr>
<td>T2DM group</td>
<td>7.8 (0.9)</td>
</tr>
<tr>
<td>Pre RYGB sNGT</td>
<td>7.5 (1.6)</td>
</tr>
<tr>
<td>Post-RYGB sNGT</td>
<td>7.9 (1.0)</td>
</tr>
<tr>
<td>Pre-RYGB sT2DM</td>
<td>8.0 (0.7)</td>
</tr>
<tr>
<td>Post-RYGB sT2DM</td>
<td>8.1 (0.8)</td>
</tr>
</tbody>
</table>

3.2.3 First strand cDNA synthesis

Total RNA was reverse transcribed using the SuperScript Vilo cDNA synthesis kit (Life Technologies). 1 μg of RNA was added to a 20μl reaction containing SuperScript III reverse transcription enzyme, random primers, RNaseOUT, Recombinant Ribonuclease Inhibitor and RNase free water.
SuperScript III is an engineered version of M-MLV reverse transcription enzyme which is not inhibited by ribosomal and transfer RNA allowing it to synthesize cDNA from total RNA. Reactions were incubated at 25ºC for 10min, then at 42 ºC for 60 minutes followed by an 85ºC 5 min incubation to deactivate the reverse transcriptase. The resulting cDNA was diluted 1:10 and stored at -20ºC prior to use in RT-qPCR reactions.

3.2.4 Analysis of gene expression with RT-qPCR

All RT-qPCR reactions were done using EXPRESS qPCR SuperMix (Life Technologies, USA) and TaqMan Gene Expression Assays (Applied Biosystems, USA). Each 20μl reaction contained 2μl of cDNA, 10μl of EXPRESS qPCR SuperMix (2X) and 1μl of TaqMan Gene Expression Assay (20X). All RT-qPCR reactions were analyzed with the ABI 7300 Real-Time PCR System (Applied Biosystems, USA) as per the thermocycling conditions presented in Table 3-2. Each sample was run in triplicate and each time a threshold cycle (Ct) was obtained using the 7300 Sequence Detection Software 1.3.1.

Table 3-2. Thermocycling conditions for RT-qPCR reactions using EXPRESS qPCR SuperMix

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Temperature (ºC)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>15 sec</td>
<td>45</td>
</tr>
<tr>
<td>Anneal/Extension and Data Collection</td>
<td>60</td>
<td>1 min</td>
<td></td>
</tr>
</tbody>
</table>

3.2.5 EXPRESS qPCR SuperMix

EXPRESS qPCR SuperMix (Life Technologies, USA) contains platinum Taq DNA polymerase, uracil DNA glycosylase, dNTPs that contain dUTP instead of dTTP and ROX reference dye. Platinum Taq DNA polymerase is a recombinant Taq DNA polymerase complexed with antibodies that blocks polymerase activity at ambient temperature. This blocking activity is removed by heat resulting in activation of the polymerase during the first denaturation step.
Essentially an automatic hot start, it increases sensitivity and specificity. dUTP incorporates uracil into any amplified DNA while uracil DNA glycosylase removes the uracil residues. This prevents the re-amplification of carryover PCR products.\textsuperscript{487} ROX dye is composed of a glycine conjugate of 5-carboxy-X-rhodamine, succinimidyl ester and is used to normalise the fluorescence signal between reactions.

### 3.2.6 TaqMan gene expression assays

Human 18S rRNA (4319413E), POLR2A (Hs 01108291_m), ENPP1/PC-1 (Hs 01054040_m1), CEACAM-1(Hs 00610438_m) and IDE (Hs 00266109_m) assays (Applied Biosystems, USA) were used. TaqMan chemistry was chosen over the more standard PCR chemistries such as SYBR green dye due to its increased specificity and decreased post-PCR processing. TaqMan Gene Expression Assays are hydrolysis probes based of work done in 1991 by Holland \textit{et al.}\textsuperscript{488} Briefly, one assay consists of two primers (900nM/primer final concentration) and one MGB probe. A minor groove binder (MGB) at the 3’ end of the probe increases the melting temperature (T\textsubscript{m}) without increasing probe length thus allowing for the design of shorter probes.\textsuperscript{489} The probe is labelled with a reporter dye at the 5’ with either 6-FAM (ENNP1, CEACAM-1, and IDE) or 6-VIC (18S) and is used at a final concentration of 250nM. Consequently, a fluorescent signal generated during amplification is used to detect a specific PCR product as it accumulates.

### 3.2.7 RT-qPCR method principle and analysis of data

Real-time quantitative PCR is based on the exponential nature of PCR where the amplicon doubles every PCR cycle,\textsuperscript{490} which can be modelled by the following equation:

\[
X_n = X_0 \times (1+E_x)^n
\]

Where \(X_n\) is the number of target molecules at cycle \(n\), \(X_0\) is the initial number of target molecules, \(E_x\) is the efficiency of target amplification and \(n\) is the number of cycles. For real-time amplification using TaqMan Gene Expression Assays, the \(X_n\) is proportional to the reporter fluorescence (R) as modelled by:
\[ R_n = R_o \times (1 + E_x)^n \]

Where \( R_o \) is the reported fluorescence at cycle \( n \) and the \( R_o \) is the initial reporter fluorescence.

The cycle threshold (Ct) is the cycle at which the fluorescent signal reaches a fixed threshold and is the main unit for calculating gene expression. The Ct value is inversely correlated to the amount of starting template meaning the lower the amount of template the longer it takes to reach threshold.

### 3.2.8 Relative quantification of gene expression

The Ct of all three replicates for a particular sample was averaged and used in subsequent data analysis as recommended by Schmittgen and Livak.\(^{491}\) Relative quantification (RQ) was used to analyze differences and changes in gene expression between groups. RQ compares the expression of the target gene in a treatment group (test sample) to that of an untreated or control group (calibrator sample). For comparisons of gene expression between individuals with and without type 2 diabetes, the NGT group was used as the calibrator sample and the T2DM group was used as the test sample. For comparisons of gene expression before and after RYGB surgery, the Pre-RYGB sNGT or sT2DM group was used as the calibrator sample and the Post-RYGB sNGT or sT2DM group was used as the test sample respectively.

Changes in gene expression were expressed as relative amount and as fold change, both of which were calculated using the \( 2^{-\Delta\Delta Ct} \) formula. The derivation of the original \( 2^{-\Delta\Delta Ct} \) formula was described in detail in the Applied Biosystems User Bulletin No.2 (P/N 4303859) and by Livak and Schmittgen.\(^{490,492} \) Using the \( 2^{-\Delta\Delta Ct} \) method, the Ct for the target gene was normalised to a reference gene Ct in each sample, which generated a \( \Delta Ct \) value. To generate the fold change, the \( \Delta Ct \) values of the calibrator sample were subtracted from the test sample which produced a \( \Delta\Delta Ct \) value that was exponentiated to generate the fold change (Figure 3.2-1).
\[
\begin{align*}
\Delta Ct_{\text{target gene}} - \Delta Ct_{\text{reference gene}} &= \Delta Ct_{\text{sample}} \\
\Delta Ct_{\text{target gene}} - \Delta Ct_{\text{reference gene}} &= \Delta Ct_{\text{calibrator}} \\
\text{Relative gene expression} &= 2^{\Delta Ct_{\text{sample or calibrator}}} \\
\Delta Ct_{\text{sample}} - \Delta Ct_{\text{calibrator}} &= \Delta \Delta Ct \\
\text{Fold Change} &= 2^{\Delta \Delta Ct}
\end{align*}
\]

Figure 3.2-1. \(2^{\Delta \Delta Ct}\) formula for calculating fold changes in gene expression between a calibrator and test sample. Exponentiating the \(\Delta Ct\) \((2^{\Delta Ct})\) of the calibrator or test sample allowed for presentation of gene expression as individual data points.

The \(\Delta Ct\) for the calibrator or test sample was also exponentiated \((2^{\Delta Ct})\) to produce relative gene expression, which allowed plotting of gene expression as individual data points. Exponentiation was necessary as the fluorescence data is recorded on a logarithmic scale \((\log_2)\) and needs to be converted to linear scale for appropriate data visualisation.

A main assumption of the \(2^{\Delta \Delta Ct}\) method is that the amplification efficiencies for both the target and reference gene are approximately equal. However there can be significant deviations in amplification efficiencies between different gene assays that may give erroneous results. TaqMan Gene Expression Assays have previously been reported to have 100±10% efficiency.\(^{493}\) To confirm this, the amplification efficiency for each TaqMan Gene Expression Assay was tested. The efficiency \((E)\) was calculated using the Ct slope method and the following equation:

\[
E_x = 10^{(-1/\text{slope})-1}
\]

cDNA transcribed from liver RNA was serially diluted and the Ct values were determined for each dilution. A plot of Ct versus log cDNA concentration was constructed. Amplification efficiency was calculated from the slope of the graphs and is presented in Table 3-3. All of the TaqMan Gene Expression Assays were approximately equal and were appropriate for use with the \(2^{\Delta \Delta Ct}\) method.
Table 3-3. TaqMan gene expression assay amplification efficiency.

<table>
<thead>
<tr>
<th>TaqMan gene expression assay</th>
<th>Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human 18S rRNA (Reference Gene)</td>
<td>98</td>
</tr>
<tr>
<td>POLR2A (Reference Gene)</td>
<td>99</td>
</tr>
<tr>
<td>ENPP1/PC-1 (Target Gene)</td>
<td>100</td>
</tr>
<tr>
<td>CEACAM-1 (Target Gene)</td>
<td>97</td>
</tr>
<tr>
<td>IDE (Target Gene)</td>
<td>91</td>
</tr>
</tbody>
</table>

3.2.9 Reference genes

Human 18S rRNA and POLR2A (Hs 01108291_m) were compared with regard to suitability as reference genes by presenting the PCR data from the biological replicates as \(2^{-\Delta C_t}\). An ANOVA was performed on the data and both reference genes were found to be suitable as there was no variation in expression between the groups being compared (p>0.1). However 18S rRNA was chosen as the reference gene due to a more normal distribution and low variance as recommend by Mane et al. (2008).494

3.2.10 Protein extraction

Protein for three of the sixteen individuals was not available prior to RYBG (2 from sNGT group and 1 from sT2DM group). Protein was extracted by pulverizing 3-4mg of frozen liver under liquid nitrogen. The resultant powder was incubated with buffer containing Complete Protease Inhibitor Cocktail (Roche), 30 mM TrisCl, 7 M urea, 2 M thiourea and 4% (w/v) CHAPS) for 45 minutes on ice, with vigorous vortexing every 15 minutes. The mixture was centrifuged at 14000g for 10 minutes to remove any insoluble material. The supernatant was taken and aliquoted further for storage at -80 ºC. Protein was quantified using Bradford reagent (Bio Rad) on a Bio-Rad Benchmark Microplate Reader.495
3.2.11 Protein resolution with E-PAGE™

The E-PAGE™ 48-well protein electrophoresis system was used to resolve protein samples on pre-cast E-PAGE 48 8% gels (20-300kDa separation range) (Life Technologies). The precast gels contain a gel matrix and electrodes enclosed in a UV-transparent cassette. They were loaded onto a specifically designed electrophoresis device that combines a base and power supply. The E-PAGE gels contain SDS so resolution was performed under denaturing conditions for 23 min.

3.2.12 Sample preparation and loading

Prior to sample and marker loading 5µl of deionized water was loaded into all wells. All samples were denatured in a total volume of 15µl. Protein lysate was made up to 15µg and mixed with E-PAGE loading buffer and β-mercaptoethanol (Sigma) (5% v/v). Samples were incubated at 70°C for 10 minutes and loaded onto the gel. To control for between/within gel variability all groups being compared were resolved in alternating lanes on the same gel. SeeBlue Plus2 Pre-stained Standard and MagicMark XP Western Protein Standard (Life technologies) were used as molecular weights to assess protein migration both during electrophoresis and western blotting respectively.

3.2.13 Electrophoretic transfer to PVDF and western blotting

Immediately after resolution, gels were equilibrated in transfer buffer (39 mM Glycine, 48 mM Tris, methanol 20% v/v) for 10 minutes. Hybond-P PVDF membrane (Amersham) was activated with methanol for 10 seconds and washed in deionized water for 5 minutes after which it was equilibrated in transfer buffer for 10 minutes. Proteins were transferred from the gel to the Hybond-P PVDF membrane with a Bio-Rad Electrophoretic transfer cell for 2-3 hours at 50V. Transfer of proteins was confirmed with Ponceau S stain. Membranes were blocked with 5% skim milk powder in TBS-Tween at 4°C overnight and then probed for 2 h at RT with ENPP1 (1:500, Santa Cruz), CEACAM-1 (1:500 Santa Cruz), IDE (1:1000 R&D) and Pan-actin (1:10000 Milipore).
Blots were then incubated with alkaline phosphatase-conjugated anti mouse antibody (Life Technologies) for 0.5 h. The chemiluminescent signal was developed using CDP-Star chemiluminescent substrate (Life Technologies) and captured on x-ray film (Kodak).

3.2.14 Stripping

Due to the scarcity of the samples, PVDF membranes were stripped once and re-probed for a different protein. Membranes were incubated in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, 62.5 mM Tris-HCL pH 6.7) at 60°C for 30 min with gentle agitation every 10 min. After rinsing with water, membranes were washed 3 times in TBS-T for 15 min at room temperature using larger volumes of wash buffer. Membranes were re-blocked overnight and re-probed the following day.

3.2.15 Relative quantification of protein abundance

The protein bands on the x-ray film were digitized by the Chemidoc XRS system (Bio Rad) and then analyzed using Quantity One software (Bio Rad). Bands were quantified and expressed as volume quantity; the sum of the intensities of the pixels within a defined volume boundary × pixel area (intensity units × mm²). Local background subtraction was used to facilitate background correction. This involves calculating separate background intensity for each volume.

Local background is calculated by taking the combined intensity of the pixels in a 1-pixel border around the volume area and dividing by the number of border pixels. This average background intensity is subtracted from each pixel contained inside the corresponding volume boundary. Any pixels inside the volume that have the same intensity as the background pixels are reduced to zero. Protein relative abundance was calculated by normalizing the intensity of the ENPP1, CEACAM-1 or IDE band to the intensity of the Actin band (loading control).
3.2.16 Statistical analysis

Statistical analysis was performed on $2^{\Delta C_{t}}$ RT-qPCR data between each study group. Consequently gene expression data was expressed as both $2^{\Delta C_{t}}$ in graphical form, whereas the fold change for significant differences was reported in text. Statistical analysis of protein abundance was performed on the value generated from normalisation of band intensity to the loading control.

Normal distribution of all groups to be compared was tested with D’Agostino-Pearson test. Non-normally distributed data was log transformed to comply with parametric test assumptions. Pre-post comparisons were carried out using a paired t-test. Student’s t-test was used to test significance between two groups. The Pearson product-moment correlation coefficient was used to test the strength and significance of association between variables. An alpha level of 0.05 was set as the significance threshold. All analysis was performed using Minitab15. Graphical visualization was performed using GraphPad Prism 5.
3.3 Results

3.3.1 Liver ENPP1 levels in diabetes

ENNP1 has been shown to negatively modulate IR activity and has been implicated in insulin resistance. Consequently, levels of ENPP1 mRNA expression and protein abundance were assayed in individuals with and without type 2 diabetes and again after resolution of insulin resistance and remission of type 2 diabetes.

Figure 3.3-1A presents mean gene expression of ENPP1 in the NGT and T2DM groups. The mean hepatic gene expression of ENPP1 was lower in the T2DM group when compared to the NGT group (Figure 3.3-1A), which is statistically significant (p=0.01, t-test). The T2DM group had a mean -1.5 (95% CI –6.2 to 2.8) fold lower ENPP1 mRNA expression than the NGT group at the time of RYGB surgery.

Figure 3.3-1C displays the western blot for ENPP1 protein abundance in NGT and T2DM groups. Loading was alternated based on disease state to avoid gel bias. The molecular weight of ENPP1 in SDS-PAGE is reported to be 110-130 kd depending on tissue type. Some part of ENPP1 reportedly exists as dimer and the 220kd band visible in Figure 3.3-1C is most likely the dimeric form of ENPP1.497

ENPP1 protein abundance data were log transformed prior to analysis with t-test in order to meet parametric test assumptions. Figure 3.3-1B shows the untransformed mean protein abundance of ENPP1. Mean ENPP1 protein abundance was lower in the T2DM group in comparison to the NGT group (Figure 3.3-1B), which was also statistically significant (p=0.03, t-test). In this study, individuals with type 2 diabetes had a mean -2 fold lower (95% CI, -3.6 to -1.3) abundance of ENPP1 than individuals with normal glucose tolerance at the time of RYGB surgery.
Figure 3.3-1. Mean ENPP1 levels in liver tissue of morbidly obese individuals. NGT; Normal glucose tolerance (n=19), T2DM; Type 2 diabetes (n=27). (A) Mean + SE ENPP1 mRNA expression presented as $2^{-\Delta Ct}$ normalised to 18S. (B) Mean + SE ENPP1 protein abundance relative to Actin. Protein was not available from two individuals in the NGT and one individual in the T2DM group. (C) Corresponding western blot for ENPP1 on all individuals who did not have a second liver biopsy. To avoid gel artefacts all samples to be compared were loaded onto the same E-PAGE™ gel, while spaces delineate different gels. Samples with an * were not included in analysis. For complete western blot film see Appendix.

3.3.2 Liver ENPP1 levels before and after RYGB surgery

ENPP1 mRNA expression and protein abundance was analysed in individuals who had normal glucose tolerance (sNGT group) or type 2 diabetes mellitus (sT2DM group) before and after RYGB surgery. ENPP1 mRNA expression data ($2^{-\Delta Ct}$) was log transformed prior to analysis with paired t-test in order to meet parametric test assumptions. Figure 3.3-2A shows the untransformed mean ENPP1 gene expression. Liver ENPP1 mRNA expression remained unchanged after RYGB surgery in the sNGT and the sT2DM group.
Figure 3.3-2B shows the untransformed mean abundance of liver ENPP1 protein before and after RYGB surgery, while Figure 3.3-2C is the corresponding western blot of ENPP1 protein for all the individuals who had a second liver biopsy after RYGB surgery. There were six pairs of liver samples from individuals with normal glucose tolerance (sNGT) while there were seven pairs of samples from individuals with type 2 diabetes (sT2DM). Pre and post RYGB samples were loaded adjacent to each other and sNGT and sT2DM pairs were alternated. ENPP1 protein abundance data was log transformed in order to meet parametric test assumptions. The sT2DM Pre-RYGB ENPP1 protein abundance tended to be lower than that of the sNGT Pre-RYGB, although this was not statistically significant (t-test). This is likely because of the small sample size in the subset of individuals who had a second liver biopsy and consequent decrease in statistical power when using the student’s t-test.

Figure 3.3-2. Liver ENPP1 gene expression and protein abundance in liver tissue of morbidly obese individuals before and after RYGB surgery (● sNGT, n=8; ○ sT2DM, n=8). (A) Mean ENPP1 mRNA expression + SE presented as $2^{-\Delta\Delta C_T}$ normalised to 18S. (B) Mean ENPP1+ SE protein abundance relative to Actin. Pre RYGB protein was not available from two individuals in the sNGT and one individual in the sT2DM group. The sT2DM group had a significant increase in the sT2DM after RYGB surgery (paired t-test). (C) Corresponding western blot for ENPP1 for individuals that had a second liver biopsy. To avoid gel artefacts all samples to be compared were loaded onto the same E-PAGE™ gel, while spaces delineate different gels. For complete western blot film see Appendix.
However, although the sNGT group had no appreciable change in ENPP1 protein levels after RYGB surgery, the sT2DM group had a statistically significant (p=0.01, paired t-test) increase in ENPP1 protein abundance (Figure 3.3-2B). The individuals who had resolution of insulin resistance and remission of type 2 diabetes following RYGB had a mean 2.7 (95% CI, 1.8 to 4.0) fold increase in ENPP1 protein abundance.

ENPP1 protein data from the sNGT and sT2DM groups were combined and log transformed prior to calculation of a Pearson product-moment correlation in order to explore any relationship with change in fasting plasma insulin concentrations and HOMA-IR. ENPP1 protein abundance negatively correlated with fasting plasma insulin concentration (Figure 3.3-3A) and HOMA-IR index (Figure 3.3-3B). Consequently, improvement in hepatic insulin sensitivity after RYGB surgery as measured by HOMA-IR was associated with increasing levels of ENPP1 protein.

Figure 3.3-3. (A) Pearson product-moment correlation of liver ENPP1 protein relative abundance (log) vs fasting plasma insulin concentration (log). (B) Pearson product-moment correlation of liver ENPP1 protein relative abundance (log) vs HOMA-IR (log).
3.3.3 Liver CEACAM-1 and IDE levels in diabetes

CECAM-1 and IDE are both involved in hepatic insulin clearance through their influence on insulin internalization and degradation respectively. Expression levels of CEACAM-1 and IDE mRNA were analysed in individuals with normal glucose tolerance (NGT group) and individuals with type 2 diabetes mellitus (T2DM group) at the time of RYGB surgery. CEACAM-1 and IDE mRNA expression data \(2^{-\Delta C_t}\) was log transformed prior to analysis with t-test in order to meet parametric test assumptions. There were no statistically significant differences between the NGT and T2DM group in CEACAM-1(Figure 3.3-4A) or IDE mRNA expression (Figure 3.3-4B) at the time of RYGB surgery.

![Figure 3.3-4 Mean + SE CAECAM-1 mRNA expression (A) and IDE mRNA expression (B) presented as \(2^{-\Delta C_t}\) normalised to 18S. NGT; Normal glucose tolerance (n=19), T2DM; Type 2 diabetes (n=27).]

Protein abundance of CEACAM-1 and IDE was also assayed at the time of RYGB surgery (Figure 3.3-5). The molecular weight of CEACAM-1 is 160-180 kd, whereas the molecular weight of IDE is reportedly 118 kd. Relative protein abundance data was log transformed prior to analysis with t-test. Although CEACAM-1 protein abundance tended to be lower in the T2DM group, this difference was not statistically significant. Similarly, there was no statistically significant difference in IDE protein abundance at the time of RYGB surgery between the NGT and T2DM group.
3.3.4 Liver CEACAM-1 and IDE levels before and after RYGB surgery

Levels of CEACAM-1 and IDE mRNA were also analysed before and after RYGB surgery in individuals who had normal glucose tolerance (sNGT group) and individuals who had type 2 diabetes mellitus (sT2DM group). CEACAM-1 mRNA expression did not change after RYGB surgery in the sNGT group or the sT2DM group (Figure 3.3-6A). Likewise, there was no statistically significant change in IDE mRNA expression in the sNGT group after RYGB surgery. However, for the sT2DM group there was a significant decrease in IDE mRNA expression after RYGB surgery (p=0.04 paired t-test) (Figure 3.3-6B). Consequently, individuals who had remission of type 2 diabetes had a mean fold change of -1.56 (95% CI -4.5 to 1.4) in IDE mRNA expression.
CEACAM-1 and IDE protein abundance was also analysed in the same individuals before and after RYGB surgery as described previously (Figure 3.3-6C and D). There were no statistically significant changes in CEACAM-1 or IDE protein abundance in the sNGT or sT2DM group.

Figure 3.3-6. Liver CEACAM-1 and IDE gene expression and protein abundance in liver tissue of morbidly obese individuals before and after RYGB surgery (● sNGT, n= 8; ○ sT2DM, n=8). (A) Mean ± SE CEACAM-1 expression presented as $2^{-\Delta\Delta Ct}$ normalised to 18S. (B) Mean ± SE IDE mRNA expression was significantly decreased in the sT2DM group after RYGB surgery (paired t-test). (C) Mean ± SE CEACAM-1 and (D) IDE protein abundance relative to Actin. Protein was not available from two individuals in the NGT and one individual in the T2DM group (E) Corresponding western blot for IDE and CEACAM-1 for individuals that had a second liver biopsy. For complete western blot film see Appendix.
3.4 Discussion

The purpose of this study was to investigate mRNA expression and protein abundance of molecules responsible for regulating insulin action in type 2 diabetes. To achieve this, levels of ENPP1, CEACAM-1, and IDE were assayed in individuals with normal glucose tolerance or type 2 diabetes before and after RYGB surgery. ENPP1 mediates IR activity, whereas CEACAM-1 and IDE are responsible for insulin internalization and degradation respectively. Any changes in levels of these molecules are likely to affect insulin action which may lead to or contribute to insulin resistance.

All individuals included in this study were morbidly obese (BMI>40 kg/m²) at the time of RYGB surgery. As expected fasting plasma glucose and HbA1c % levels were normal in individuals with normal glucose tolerance. However, individuals with type 2 diabetes had significantly higher fasting plasma glucose and HbA1c % values, which returned to normal after RYGB surgery (Figure 2.3-3). Although insulin resistance was present in the majority of individuals included in the study, those with type 2 diabetes had significantly higher fasting plasma insulin concentrations and HOMA-IR values, than those with NGT. RYGB surgery was associated with improved insulin sensitivity in both groups of individuals, with normalisation of fasting plasma insulin concentrations and HOMA-IR values being associated with remission of type 2 diabetes (Figure 2.3-2 and 2.3-3).

ENPP1, CEACAM-1, and IDE expression was compared between individuals grouped according to their glucose tolerance status, and hence insulin resistance. Individuals with normal glucose tolerance (NGT and sNGT groups) were used as controls.

ENPP1 inhibits insulin signalling by directly binding the α-subunit of the insulin receptor. Evidence of elevated ENPP1 protein levels in muscle, adipose and skin of insulin-resistant individuals has led to the hypothesis that this molecule may be integral to the development of insulin resistance. If ENPP1 contributes to the development of insulin resistance then it should be overexpressed in individuals with advanced insulin resistance (T2DM group) and decrease with resolution of insulin resistance (sT2DM group).
The principal role of CEACAM-1 in insulin action is to mediate insulin internalization, whereas IDE is responsible for degrading a number of substrates including insulin. Insulin internalization and degradation are both required for effective insulin clearance at the liver, which others have shown to increase after RYGB surgery in individuals who have a remission of diabetes. Changes in mRNA expression and protein content of these genes after the remission of type 2 diabetes (sT2DM group) may shed light on the mechanisms involved in hepatic insulin clearance at the liver.

3.4.1 ENPP1

Although ENPP1 may contribute to the pathology of insulin resistance, conflicting evidence has been reported in the literature. Overexpression of ENPP1 protein in the muscle and liver of animal models leads to development of insulin resistance. However, in an animal model of diabetes there was no difference in ENPP1 muscle and liver content in comparison to control animals. Even though some experimentally produced type 2 diabetes animal models resulted in increased ENPP1 content others did not. Furthermore, there was no change in muscle ENPP1 content after bariatric surgery where there had been a measured increase in insulin sensitivity.

There have been no studies reporting ENPP1 levels in the liver of human individuals with or without type 2 diabetes. Considering the implications involved in pathological overexpression of ENPP1, this study sought to address this mechanism. Although we found ENPP1 mRNA expression to be lower in individuals with type 2 diabetes (Figure 3.3-1A), there was no change in expression after remission of diabetes. This may be due to variable protein turnover as studies have shown that in liver cells insulin can increase ENPP1 protein abundance without changing its gene expression. Nonetheless, there was a clear association between ENPP1 protein abundance and type 2 diabetes. One of the key findings was that liver ENPP1 protein abundance was 2 fold lower in individuals with type 2 diabetes in comparison to individuals with normal glucose tolerance (Figure 3.3-1B). Furthermore, individuals who had remission of type 2 diabetes had a 2.7 fold increase in ENPP1 protein expression (Figure 3.3-2).
This data differs from previous studies which showed that ENPP1 levels in muscle and adipose tissue were inversely correlated with insulin sensitivity.\textsuperscript{167, 168, 504} We suspect that the disagreeing evidence reported in the literature and our own data presented here is due to the varied roles muscle, fat, and liver tissues have in regulating insulin action.

The liver has a crucial role in regulating the systemic insulin concentration by hepatic insulin clearance.\textsuperscript{56} It is the first organ to receive large amounts of insulin released from the pancreas during the first phase response (1000-5000 pmol/l) and removes $\sim$50\% of it from the bloodstream.\textsuperscript{62, 63} Considering the liver extracts and recycles supraphysiological levels of insulin during first phase insulin secretion, preventing overstimulation of metabolic pathways may be necessary for normal glucose homeostasis. Evidence for a possible insulin self-desensitisation mechanism was first provided by Menzaghi et al. (2003) who found that insulin induces a rapid increase in ENPP1 at the cell membrane in liver cells.\textsuperscript{503} Additionally, individuals with insulin receptor or IRS-1 mutations resulting in defunct insulin signalling had a significant reduction in ENPP1 activity and content.\textsuperscript{505}

In this study, ENPP1 protein abundance was increased with an improvement in insulin sensitivity in individuals who had a remission of type 2 diabetes and was inversely correlated with both fasting plasma insulin concentration and HOMA-IR (Figure 3.3-3). The increase in ENPP1 levels after an improvement in insulin sensitivity is consistent with the hypothesis that ENPP1 acts as a part of a hormone self-desensitization mechanism. However the fact that it occurs only in individuals who have a remission of type 2 diabetes is interesting and could be explained by the nature of insulin secretion and its abrogation in type 2 diabetes.

The first-phase insulin response is triggered by an increase in glucose concentration and markedly increases the insulin pulse mass.\textsuperscript{40} One of the earliest defects in individuals who develop type 2 diabetes is a decreased first phase insulin pulse mass.\textsuperscript{40, 48, 52} Restoration of the first-phase insulin response after gastric bypass surgery in individuals who have a normalisation of insulin sensitivity and remission of type 2 diabetes has been documented.\textsuperscript{471} Consequently, the increase in ENPP1 levels in individuals who have remission of type 2 diabetes after gastric surgery could be a response to the return of the first phase insulin response.
If this is the explanation then ENPP1 is unlikely to be contributing to the development of insulin resistance at the liver in morbidly obese individuals but instead may be a compensatory response for increased insulin sensitivity after RYGB surgery.

However as this study does not address the K121Q polymorphism it cannot be concluded that ENPP1 does not have a role in liver insulin resistance. Recently it has been shown that the relatively rare Q121 variant has a greater inhibitory effect on insulin receptor than the more common K121 variant in insulin target cells\(^{506}\) and may interact with adiposity to modulate glucose homeostasis \(^{507}\). But the question of whether there is a direct link with type 2 diabetes is yet to be answered. Although the K121Q polymorphism has been shown to be associated with type 2 diabetes in meta-analysis \(^{508}, 509\) there is some conflicting evidence \(^{510}, 511\) making it difficult to conclusively link it with type 2 diabetes.

### 3.4.2 CEACAM-1 and IDE

Transgenic mice with a functional mutation in liver CEACAM-1 develop hyperinsulinaemia as a result of impaired insulin clearance.\(^{65}\) This leads to secondary insulin resistance, abnormal glucose tolerance, increased fatty acids, hepatic steatosis and visceral adiposity.\(^{65}, 70\) Consequently the ability of CEACAM-1 to regulate insulin internalisation and hence clearance has been implicated in the pathogenesis of insulin resistance and type 2 diabetes.

In this study CEACAM-1 mRNA and protein abundance did not change in varying states of insulin resistance nor did it change after an increase in insulin sensitivity following RYGB. Although CEACAM-1 protein content has been shown to be decreased in liver of individuals with severe obesity or liver steatosis, it did not differ between individuals with or without type 2 diabetes.\(^{512}\) This is in accordance with the data presented here, suggesting that CEACAM-1 may not be directly involved in the pathogenesis of type 2 diabetes.

IDE has also been implicated in the pathogenesis of insulin resistance. In human studies, two IDE polymorphisms were associated with an increased risk of type 2 diabetes,\(^{74}\) whereas mice lacking IDE developed hyperinsulinaemia leading to
progressive insulin resistance and ultimately glucose intolerance. In this study there were no significant differences in IDE gene expression or protein content between individuals with normal glucose tolerance or type 2 diabetes. However, there was a significant decrease in IDE mRNA expression in individuals who had a remission of type 2 diabetes (Figure 3.3-6B). A study by Pivovarova et al. (2009) showed that treating liver cells with high glucose concentrations can cause an increase in IDE mRNA expression, which was associated with a loss of insulin-induced changes in IDE activity. This provides a possible explanation for our findings.

Although the data presented here does not address IDE activity, reduced IDE mRNA expression was only seen in those individuals that had a significant decrease in fasting plasma glucose levels (Table 2-3). Taken together these findings suggest that hyperglycaemia may provoke a disturbance in IDE activity in type 2 diabetes which may lead to inefficient hepatic insulin clearance causing an increase in systemic insulin concentration. This presents a potential molecular mechanism responsible for driving the progressive insulin resistance seen in type 2 diabetes. However, in our studies, IDE mRNA and protein did not have a statistically significant correlation with fasting plasma glucose concentrations, suggesting the mechanisms by which high glucose concentrations affect IDE-mediated degradation of insulin are complex. Considering IDE also has a major role in degrading amyloid-β protein it is reasonable to assume its activity may be regulated by components of both pathways.

In conclusion, the results from this study demonstrate a reverse ENPP1 pattern to that previously described in muscle and adipose tissue of insulin-resistant individuals. They suggest that ENPP1 acts as a desensitizer under conditions of insulin sensitivity rather than being an important contributor to insulin resistance in the liver. Furthermore, although individuals who undergo RYGB surgery have an increase in hepatic insulin clearance, data presented here suggest that this is not associated with changes in expression of CEACAM-1 or IDE.
CHAPTER FOUR: Liver insulin receptor isoforms and their role in the pathogenesis of type 2 diabetes
4.1 Introduction

Insulin signalling occurs primarily through the insulin receptor.\textsuperscript{129, 130} In the 1980s the discovery of the IR isoforms\textsuperscript{358} led to extensive research into the molecular mechanism regulating insulin action. Since then, a massive body of evidence has been gathered regarding post receptor events of the insulin signalling pathway. Despite this, the regulation of these outcomes by the IR isoforms in insulin target tissue, such as the liver, is still unclear.

Alternative splicing of exon 11 in the \textit{INSR} gene generates two different protein isoforms, IR-A (without exon 11) and IR-B (with exon 11).\textsuperscript{358} Because of its mitogenic characteristics IR-A predominates in developmental tissue such as fetal cells and in pathological conditions such as cancer.\textsuperscript{367, 368, 382} Conversely, IR-B is expressed in differentiated adult cells and is predominant in insulin sensitive tissues which regulate glucose homeostasis.\textsuperscript{365, 366} The high affinity for growth promoting ligands\textsuperscript{367, 514} and a faster internalisation rate of the IR-insulin complex\textsuperscript{373, 374, 390} are molecular mechanisms that are thought to account for the mitogenic properties of IR-A activation. On the other hand, the slower internalisation rate\textsuperscript{390} and stronger activation of the IR tyrosine kinase\textsuperscript{376-378} accounts for the metabolic function of IR-B activation by insulin. Signalling through IR receptor isoforms is further complicated by the formation of hybrid receptors. IR-A, IR-B and IGF-IR proreceptors generate heterodimeric hybrids that have been shown to reduce the number of insulin binding sites and may contribute to the pathogenesis of insulin resistance.\textsuperscript{404, 407}

Multiple lines of evidence point to the liver being the vital organ in the development of type 2 diabetes. First, unregulated hepatic gluconeogenesis is thought to be a critical component of the fasting hyperglycaemia in late stage type 2 diabetes.\textsuperscript{19, 21, 23, 515} Second, tissue specific IR knockout studies in mice suggest that of the three insulin target tissues involved in glucose homeostasis, the liver may be of primary importance.\textsuperscript{111, 116, 123, 516} Neither adipose nor muscle insulin receptor knockout mice have perturbations in glucose and insulin levels.\textsuperscript{112, 114, 517} But liver insulin receptor knockout mice develop both hyperglycaemia and hyperinsulinemia which ultimately leads to diabetes.\textsuperscript{116, 123} The liver also appears to be a vital organ in the remission of type 2 diabetes after RYGB surgery. Liver insulin resistance as measured by HOMA-
IR has been shown to rapidly resolve after RYGB surgery, in the same timeframe as remission of type 2 diabetes, whereas peripheral insulin resistance seems to only improve sometime later and after substantial weight loss.\textsuperscript{448, 476, 477, 481}

Although there is conflicting evidence in the literature\textsuperscript{418, 419, 421, 423, 424, 426, 518} altered relative expression of the two IR isoforms has previously been implicated in the pathogenesis of type 2 diabetes. Most studies analysing these isoforms have involved skeletal muscle and adipose tissue but relatively little work has been done in human liver. Because there is reason to believe the liver may be the key organ in the development of type 2 diabetes, we have chosen to investigate whether the insulin receptor isoforms change in liver tissue after remission of type 2 diabetes. Additionally, we explore whether any changes in the ratio of the two isoforms has implications for liver insulin signalling.

\textbf{4.1.1 Experimental strategy}

- IR protein content and isoform gene expression was assayed using western blot and RT-qPCR in the NGT, T2DM, sNGT and sT2DM groups.
- IR protein content and isoform gene expression in Hep G2 cells treated with supraphysiological levels of insulin was assayed using western blot and RT-qPCR.
- Attempted siRNA-mediated knockdown of IR-A or IR-B to investigate their functional characteristics regarding insulin signalling.
- Overexpression of IR-A or IR-B in Hep G2 cells and assaying activation of AKT (Ser\textsuperscript{473} phosphorylation) with western blot and levels of \textit{PCK1} mRNA transcription using RT-qPCR after insulin treatment.
4.2 Research Design and Methods

The cohort described in Chapter 2 was used in this study. This cohort included individuals with normal glucose tolerance (NGT group, n= 19), and type 2 diabetes (T2DM group, n=27). In addition we made use of liver tissue obtained from a subset of individuals who were re-operated on subsequent to the RYBG, for unrelated reasons, who had either normal glucose tolerance (sNGT, n=8) or type 2 diabetes (sT2DM, n=8). The following methods pertain to analyses of IR-A and IR-B isoform mRNA expression and IR protein content in these individuals.

4.2.1 Analysis of IR-A and IR-B mRNA expression using RT-qPCR

For RNA extraction/quality control, first strand cDNA synthesis and thermocycling conditions refer Chapter 3 Research Design and Methods. Briefly, all RT-qPCR reactions were done using EXPRESS qPCR SuperMix (Life Technologies) and TaqMan Gene Expression Assays (Applied Biosystems, USA). Single stranded cDNA was analyzed with the ABI 7300 Real-Time PCR System (Applied Biosystems, USA). Each sample was run in triplicate and each time a threshold cycle (Ct) was obtained using the 7300 Sequence Detection Software 1.3.1. The average of all three replicates for a particular sample was calculated and used in subsequent data analysis.

Custom designed IR-A (A127515) and IR-B (A1Q7CX) assays were used (Applied Biosystems, USA). IR-A and IR-B differ by the presence or absence of a 36 base pair sequence (Exon 11) making it difficult to assay using RT-qPCR with conventional chemistries such as SYBR green. Because of their increased specificity, Custom TaqMan Gene Expression Assays were designed and produced specifically for this experiment. For both assays the forward and reverse primers were identical. Forward Primer: 5’-GATTACCTGCACAACGTGT-3’; Reverse Primer: 3’-GCCAAGGGACCTGCCTT-5’.
The MGM probe for the TaqMan assay for IR-A crossed the exon 10/12 boundary while the MGM probe for IR-B crossed the exon 11/12 boundary (Figure 4.2-1). Amplification efficiency of IR-A and IR-B probes were assayed using the Ct slope method and $E_x = 10^{(-1/\text{slope})-1}$ equation as described in Chapter 3 Research Design and Methods. Amplification efficiency for IR-A was 100% while the efficiency for IR-B was 98%. Changes in gene expression were expressed as relative amount normalised to a reference gene and as fold change, both of which were calculated using the RQ method ($2^{-\Delta\Delta Ct}$). Eukaryotic 18S rRNA (4319413E, Applied Biosystems) was used as the reference gene.

**4.2.2 Analysis of IR protein abundance using E-PAGE electrophoresis and western blotting**

Insulin receptor protein abundance was analysed in human liver tissue as described in detail in Chapter 3 Research Design and Methods. Briefly, 15 µg of protein per sample was resolved by E-PAGE electrophoresis (Life Technologies) and transferred to Hybond-P PVDF membrane (Amersham). Membranes were blocked with 5% skim milk powder in TBT-T at 4°C overnight and then probed for 2 h at room temperature with Anti-Insulin Receptor β-subunit antibody (Clone CT-3, 1:500, Millipore) Blots were then incubated with alkaline phosphatase-conjugated anti mouse antibody (Life Technologies) for 0.5 h. The chemiluminescent signal was developed using CDP-Star chemiluminescent substrate (Life Technologies) and captured on x-ray film (Kodak). The protein bands on the x-ray film were digitized by the Chemidoc XRS system (Bio...
Rad) and then analyzed using Quantity One software (Bio Rad). Bands were quantified and expressed as volume quantity; the sum of the intensities of the pixels within a defined volume boundary × pixel area (intensity units × mm²). Local background subtraction was used to facilitate background correction. Insulin Receptor protein relative abundance was calculated by normalizing the intensity of the IR to the intensity of the Actin band.

4.2.3 Functional studies of insulin receptor and its isoforms in cultured liver cells

The Hep G2 cell line was purchased from the American Type Culture Collection (ATCC number: HB-8065, Manassas, VA, USA). This cell line was derived from the liver tissue of a 15 year old male with differentiated hepatocellular carcinoma. Hep G2 cells are adherent epithelial-like cells that grow as a monolayer and in small aggregates.

4.2.3.1 Propagation and storage

Hep G2 cells were maintained in a 500cm² Square TC-Treated Culture Dish (Corning) in Dulbecco’s modified eagle medium (DMEM) (Life Technologies), supplemented with 10% fetal calf serum (FCS) (Life Technologies, NZ) and antibiotic/antimycotic (penicillin 100units/ml, streptomycin 100µg/ml and fungizone 0.25µg/ml - Life Technologies) at 37°C and 5% CO₂. From this point forward the acronym DMEM denotes DMEM supplemented with 10% FCS and antibiotic/antimycotic.

At 70-80% confluence, cells were detached with Trypsin/EDTA solution (TE) (Life Technologies). The trypsin was inactivated with an equal volume of DMEM and centrifuged at 1100g for 5 minutes. The cell pellet was washed with DMEM to remove residual TE solution and re-centrifuged at 1100g for 5 minutes. The washed pellet was re-suspended in 6mL of DMEM and then combined with a further 6mL of DMEM with 10% (v/v) DMSO, which was added dropwise to the cell suspension for a final volume of 12mL and a 5% (v/v) DMSO concentration.
1mL of this cell suspension was aliquoted into 1.5mL cryovials (Nalgene) and cooled to -80ºC at a rate of 1ºC/min in a Cryo 1ºC freezing container (Nalgene) containing 250mL isopropanol. Cells were transferred to a liquid nitrogen dewar for long term storage in liquid nitrogen.

4.2.3.2 In Vitro experiments

Depending on the amount of cells need, 1-2 cryovials were brought out of storage and defrosted until the cell mixture reached an ice slush state. At this point the cell slurry was transferred to either a 25cm² (Corning) or a 75cm² (Labcon) TC-Treated angled neck flask containing DMEM warmed to 37ºC. Cells were left to attach for 6-12 hours after which media was refreshed. The following day, cells were trypsinized, counted in a Neubauer Counting Chamber and used in subsequent experiments.

4.2.3.3 RNA extraction from cultured cells

For cultured cell monolayers, media was removed and cells were washed in 1mL phosphate-buffered saline (PBS). 800µL of TRIzole® (Life Technologies) was added per well, homogenised and incubated at room temperature for 5 min before transfer to RNAase/DNAase free eppendorf tubes. RNA was extracted as described in Chapter 3 Research Design and Methods.

4.2.3.4 RNA quantification

RNA quantity was measured using a NanoDrop ND-1000 Spectrophotometer. The absorbance of the RNA sample was measured between 260 and 280nm. An A260 reading of 1.0 is equivalent to 40μg/ml of RNA. The concentration of nucleic acid was determined using the Beer-Lambert law which predicts a linear change in absorbance with concentration. The absorbance maximum of RNA is at 260nm and the ratio of the absorbance at 260 and 280nm gives an indication of RNA purity. Only RNA with OD_{260}/OD_{280} ratio greater than 1.8 was used.
4.2.3.5 RT-qPCR of IR-A/IR-B and PCK1 in Hep G2 cells

The principle and most of the methodology was identical to the methods described in Chapter 3 Research Design and Methods unless otherwise stated.

Total RNA was reverse transcribed using the iScript Advanced cDNA Synthesis kit for RT-qPCR (Bio Rad). 1 µg of RNA was added to a 20µl reaction containing iScript MMLV reverse transcription enzyme, random primers, dNTPs, oligo(dT), buffer and RNase inhibitor. Reactions were incubated at 42ºC for 30min, then at 85ºC for 5 minutes. The resultant cDNA was diluted 1:10 and stored at -20ºC prior to use in RT-qPCR reactions.

All RT-qPCR reactions were done using TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (Applied Biosystems, USA). Custom designed IR-A (A127515)/IR-B (A1Q7CX) assays and PCK1 TaqMan Gene Expression Assay on Demand (Hs00159918_m1) were used (Applied Biosystems). Single stranded cDNA was analyzed with the ABI 7300 Real-Time PCR System (Applied Biosystems USA). Each sample was run in duplicate and each time a threshold cycle (Ct) was obtained using the 7300 Sequence Detection Software 1.3.1. The average of replicates for a particular sample was calculated and used in subsequent data analysis.

4.2.3.6 Protein extraction

For cultured cell monolayers, media was removed and cells were washed in 1mL PBS. Cells were lysed in the well by adding 100µl Laemmli buffer (62.5mM Tris-Hcl, 2% SDS, 25% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue) containing Complete Protease Inhibitor Cocktail (Roche) and Phosphatase Inhibitor Cocktail C (Santa Cruz). The cells were homogenised and incubated for 5 minutes. The lysate was transferred to an eppendorf tube and incubated at 100ºC for 5 minutes. Centrifugation was performed at 14,000 g for 10 minutes at 4ºC to remove insoluble debris. The supernatant was aliquoted and stored at -80ºC prior to SDS-PAGE.
4.2.3.7 SDS-PAGE

SDS-PAGE gels were cast using the Mini-Protean Tetra Casting Module (Bio Rad). Each gel had 10 wells with a 4% stacking layer (4% Acrylamide/Bis, 0.125M Tris, pH 6.8, 0.1% SDS, 0.1% TEMED, 0.05% APS) and a 10% resolving layer (10% Acrylamide/Bis, 0.375M Tris, pH 8.8, 0.1% SDS, 0.1% TEMED, 0.05% APS). Samples were resolved using the Mini-PROTEAN 3 cell (Bio Rad). 20µL of lysate was added to each well and run at 100V until the dye front reached the resolving layer at which point the voltage was increased to 160V until the dye front reached the end of the gel.

4.2.3.8 Electrophoretic transfer to PVDF and western blotting

Immediately after resolution gels were equilibrated in transfer buffer (39 mM Glycine, 48 mM Tris, methanol 20% v/v) for 10 minutes. Hybond-P PVDF membrane (Amersham) was activated with methanol for 10 seconds and washed in deionised water for 5 minutes after which it was equilibrated in transfer buffer for 10 minutes. Proteins were transferred from the gel to the Hybond-P PVDF membrane with a Bio-Rad Mini Trans-Blot Cell for 2-3 hours at 50V. Transfer of proteins was confirmed with Ponceau S stain. The membrane was cut according to molecular weight which allowed for probing of different proteins simultaneously. Membranes were blocked with 5% skim milk powder in TBS-Tween at 4°C overnight and then probed for 2 h at RT with primary antibody. Blots were then incubated with alkaline phosphatase-conjugated anti mouse or rabbit antibody (Life Technologies) for 0.5 h. The chemiluminescent signal was developed using CDP-Star chemiluminescent substrate (Life Technologies) and captured on x-ray film (Kodak).

4.2.3.9 Stripping

PVDF membranes were only stripped once and re-probed for a different protein. Membranes were incubated in stripping buffer (100 mM β-mercaptoethanol, 2% (w/v) sodium dodecyl sulphate, 62.5 mM Tris-HCL pH 6.7) at 60°C for 30 min with gentle agitation every 10 min. After rinsing with water, membranes were washed 3 times in TBS-T for 15 min at room temperature using larger volumes of wash buffer. Membranes were re-blocked overnight and re-probed the following day.
4.2.4 Transient Knockdown of IR-A and IR-B isoform expression using siRNA

Small interfering RNAs (siRNA) ~ 21 to 23 nucleotides in length induce sequence-specific gene silencing. The efficiency of gene silencing is dependant on the susceptibility of target transcripts to siRNA-mediated RNA-induced silencing complex (RISC) activity. Typically two or more siRNA sequences with high prediction scores against the target sequence are chosen for knockdown of the target gene. However due to the short sequence of exon 11 (36 base pairs) the design of completely different siRNA sequences was not possible. The GeneAssist Custom siRNA Builder (Applied Biosystems) was used to design Silencer Select siRNA for IR-A and IR-B isoform by providing the target sequence. Pre designed and validated Silencer Select siRNA (Applied Biosystems) for Insulin Receptor (s7477 and s7479) were used for comparison.

Table 4-1. IR-A and IR-B siRNA sequence

<table>
<thead>
<tr>
<th>Target Sequence Input</th>
<th>SiRNA Sequence (5’-&gt;3’, sense)</th>
<th>SiRNA Sequence (5’-&gt;3’, antisense)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IR-B siRNA (s239121)</td>
<td>AAAAACCUUCUCAGGCACUGGU GCCGAGGACCCCUAG (insr exon 11)</td>
<td>AAACCUCUUCAG GCACUGGtt</td>
</tr>
<tr>
<td>IR-B siRNA (s239120)</td>
<td>AAAAACCUUCUCAGGCACUGGU GCCGAGGACCCCUAG (insr exon 11)</td>
<td>AACCUCUUCAGG CACUGGtt</td>
</tr>
<tr>
<td>IR-A siRNA (s244179)</td>
<td>AACGUUGGUUUUCGUCGCCAGGG AGUCCUCGUUUAGGAAGA (insr exon 10/12)</td>
<td>AGGCCAUCUCGGG AAACGCAtt</td>
</tr>
</tbody>
</table>

4.2.4.1 siRNA transfection

Hep G2 cells were transfected using RNAiMAX (Life Technologies), a cationic lipid transfection agent. There are two different protocols for transfecting cells in culture, forward and reverse transfection. Traditionally forward transfection has been more common. In forward transfection cells are first plated in multi-well plates and left to adhere. Transfection complexes are prepared and added the following day. With the advent of siRNA and high throughput screening, reverse transfection has been developed.
In this protocol transfection complexes are prepared and added to multi-well plates after which cells and media are added. Reverse transfection is quicker than forward transfection and has been found to increase transfection efficiency in Hep G2 cells. Commercial companies such as Life Technologies who provide transfection agents such as Lipofectamine and RNAiMAX state that Hep G2 cells must be reverse transfected with RNAiMAX (Life Technologies) to achieve adequate transfection efficiency. This is likely due to the increased cell surface area exposed to the transfection complex in suspension in comparison to monolayer. For this reason, reverse transfection with RNAiMAX was used to transfect siRNA into Hep G2 cells.

4.2.4.2 Reverse transfection of siRNA

Transfection complexes were prepared in sterile RNAase/DNAase eppendorf tubes in master mix format for each siRNA and then added to a 24-well TC-Treated plate (Labcon). The volumes used in the following description are in per well format. The transfection complex volume for each well was 100µL. Opti-MEM (Life Technologies), a modified DMEM media with reduced serum content, was used to dilute both the siRNA and RNAiMAX (Life Technologies). siRNA (1.5µL of 20mM stock, 50nM final well concentration) was diluted in 50µL of Opti-MEM and mixed gently. RNAiMAX was diluted in 50µL of Opti-MEM and combined with the siRNA dilution. The transfection complex was incubated at room temperature for 20 minutes. During this incubation, Hep G2 cells were trypsinized and centrifuged at 1100 g for 5 minutes. The cell pellet was re-suspended in DMEM without antibiotic/antimycotic and counted in a Neubauer Counting Chamber.

The cell suspension was diluted to 100 cells/µL. Transfection complex was added to the bottom of each well after which 500µL of cell suspension was added to each well for a final concentration of 4 x 10⁴ cells/well. Cells were incubated at 37°C and 5% CO₂ for 24 hours after which media was refreshed. Gene and protein knockdown was assayed 48 and 72 hours after transfection respectively using RT-qPCR and western blotting as described previously. All knockdown experiments were done in duplicate. Experimental controls were run in parallel which included negative control (scramble siRNA, Applied Biosystems) and mock transfection.
Silencer Select GAPDH (Applied Biosystems) was used to optimize transfection conditions and as a positive control.

### 4.2.5 IR-A and IR-B Isoform overexpression vector

pcDNA3.1+ vector containing IR-B cDNA (a kind gift from Ronald Kahn Lab, Joslin Diabetes Centre, Boston, USA) was used to over express IR-B isoform in Hep G2 cells. The vector was received dried on Whatman #1 filter paper from Prof. Conan Fee at the Biomolecular Interaction Centre, Christchurch, New Zealand. To retrieve the vector the marked circular area that contained it was cut and inserted into a 1.5mL RNAase/DNAase free eppendorf tube. 100µL of TE buffer (10mM Tris, 1 mM EDTA, pH 8.0) was added after which the tube was vortexed and incubated at room temperature for 5 minutes. After the incubation it was vortexed again and centrifuged at 1000g for 1 minute. The resulting supernatant was used to transform *E. coli* for propagation of the vector.

#### 4.2.5.1 Transformation

Prior to thawing cells, 10µL of vector was added to an eppendorf tube and chilled on ice. Subcloning Efficiency DH5α (*E. coli*) Competent cells (Life Technologies) were thawed on ice and gently mixed with pre-chilled pipette tips. 50µL was aspirated and added to either the tube containing the vector or to an empty pre-chilled 1.5mL eppendorf tube for storage at -80°C. The cells were gently mixed and incubated on ice for 30 minutes. The cells were then heat shocked in a 42°C water bath for exactly 20 seconds and immediately cooled on ice for 2 minutes.

Following the 2 minute cooling period, 950µL of pre-warmed (37°C) SOC medium (tryptone 10g/L, yeast extract 5g/L, NaCl 5g/l, 20mM glucose, Sigma Aldrich) was added and incubated at 37°C for 1 hour at 225 rpm. After the 1 hour incubation with SOC medium, 50 and 100µL of bacterial suspension was spread onto two different LB agar (Life Technologies) plates containing 50µg/mL ampicilin (Sigma Aldrich) and incubated at 37°C overnight. The following day bacteria were evenly spread across the plate in isolated colonies.
4.2.5.2 Vector propagation and extraction

Several colonies were picked with a sterile pipette tip and placed in separate 50mL tubes (BD Falcon) containing 5mL LB medium (Sigma Aldrich) with 50µg/mL ampicillin and incubated at 37ºC overnight at 225rpm. The following day, 500µL of cell suspension was diluted with 500µL of LB medium containing 50% glycerol (final concentration 25%) and stored at -80ºC as stock.

The remaining bacterial suspension was centrifuged at 1500g for 5 minutes and the plasmid was extracted from the resulting pellet using the EZNA Plasmid Miniprep Kit I (Omega Bio-Tek). This kit is based on the DNA extraction method established by Birnboim et al. (1979) in which alkaline conditions are used to selectively precipitate and remove chromosomal bacterial DNA whilst retaining plasmid DNA. Additionally HiBind columns that can reversibly bind DNA are used for efficient purification. Plasmid DNA that was bound to the HiBind column was eluted with TE buffer. Plasmid DNA was quantified using the NanoDrop ND-1000 Spectrophotometer and confirmed using both enzyme digestion and PCR.

4.2.5.3 Plasmid enzyme digestion

The IR-B cDNA size was briefly confirmed by enzyme digestion. pcDNA3.1 IR-B plasmid was digested with XbaI and HindIII (Fermentas) according to the following protocol; 13.75µL RNAase/DNAase free H₂O, 2µL HindIII, 1µL XbaI, 2µL Tango buffer (10x stock) and 1.25ul DNA (800ng/µL) was added to a tube and incubated at 37ºC for 2.5 hours. The digest was separated on a 1% TAE Agarose gel and resolved using a Mini SUB Gel GT electrophoresis chamber (Bio Rad) at 5V/cm.

HyperLadder I (Bioline) was used as a DNA marker. Bands were visualised after staining with Ethidium Bromide (Sigma Aldrich) under UV light and images were recorded using the Chemidoc XRS system (Bio Rad).
4.2.5.4 PCR of linear plasmid

PCR was performed on linear plasmid DNA. The primer pairs for PCR of the INSR gene were designed with the Gene Link Oligo Explorer software using the consensus coding sequence (CCDS) obtained from the NCBI reference sequence database. The primers were designed to target the 5’ and 3’ end of the sequence. Further nucleotides were added to create restriction sites for HindIII and XbaI with a view to cloning the IR-A isoform into the pcDNA 3.1 vector. The forward primer sequence was further manipulated to add a Kozak sequence for increased translational efficiency. Primer sequences are shown in Figure 4.2-3.

<table>
<thead>
<tr>
<th>Forward Primer (HindIII)</th>
<th>Reverse Primer (XbaI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INSR CCDS:</strong> 5’-ATG GCC ACC GGG GGC CGG CGGGGGGCG</td>
<td><strong>INSR CCDS:</strong> CTG ACC TTG CCT CGG TCC AAT CCT TCC TAA-3’</td>
</tr>
<tr>
<td><strong>Primer:</strong> 5’-CAT AAG CTT GCC ATG GCC ACC GGG GGC CGG-3’</td>
<td><strong>Primer:</strong> 3’-CTGG AAC GGA GCC AGG TTA GGA AGG ATT AGA TCT CG-5’</td>
</tr>
<tr>
<td></td>
<td>5’-GC TCT AGA TTA GGA AGG ATT GGA CGG AGG CAA GGT C-3’</td>
</tr>
</tbody>
</table>

Figure 4.2-3. Forward and reverse primer sequences for confirmation of IR-B cDNA clone and further cloning of IR-A cDNA designed using the insulin receptor consensus coding sequence (INSR CCDS). Italicized nucleotides are enzyme restriction sites.
PCR was performed using BioMix Red PCR master mix (Bio Line). Due to the high G/C content in the first 50bp of the INSR gene (88%) and the forward primer (66%) DMSO was used as an additive at a final concentration of 5%. To prevent spurious priming PCR cycling conditions were optimised using the Touchdown technique as reported by Don et al. (1991). The nearest-neighbour theory of DNA duplex stability was used to calculate melting temperature ($T_m$). Briefly, overall duplex stability or otherwise melting temperature of an oligonucleotide can be predicted from the primary sequence using the stability and temperature dependant behaviour of every dinucleotide pair in the oligo. Using Gene Link Oligo Explorer software, the $T_m$ was estimated to be 81°C and 75°C for the forward and reverse primer respectively.

Each 50µL PCR reaction contained 18µL RNAase/DNAase free H₂O, 2µL DNA, 1.25µL forward and reverse primer (20µM stock, 0.5µM final concentration), 2.5µL DMSO and 25µL BioMix Red master mix. Thermocycling parameters are shown in Table 4-2. The PCR product was separated on a 1% TAE agarose gel and resolved using a Mini SUB Gel GT electrophoresis chamber (Bio Rad) at 5V/cm. HyperLadder I (Bioline) was used as a DNA marker. Bands were visualised after staining with Ethidium Bromide (Sigma Aldrich) under UV light and images were recorded using the Chemidoc XRS system (Bio Rad).

Table 4-2. Touchdown PCR thermocycling parameters for insulin receptor.

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95°C</td>
<td>5 min</td>
<td>1</td>
</tr>
<tr>
<td>Touchdown Phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
<td>17</td>
</tr>
<tr>
<td>Annealing</td>
<td>85°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>4.30 min</td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>95°C</td>
<td>30 sec</td>
<td>17</td>
</tr>
<tr>
<td>Annealing</td>
<td>69°C</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>4.30 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4°C</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>
4.2.5.5 Attempted cloning of IR-A

Hep G2 cells were grown in a 25cm² TC-treated tissue culture flask (Corning) in DMEM (Life Technologies), supplemented with 10% foetal calf serum (FCS) (Life technologies, NZ) at 37°C and 5% CO₂. RNA was extracted using TRIzole® as described previously. First strand synthesis was performed using SuperScript III First-Strand Synthesis System for RT-qPCR (Life Technologies). The reaction contained 5µg RNA, 2.5µM Oligo(dT)₂₀, 10mM dNTP mix, 5mM MgCl₂, 10mM DTT, 40U RNase OUT, and 200U SuperScript III. Reactions were incubated at 50°C for 50 min and terminated at 85°C for 5 min. cDNA synthesised from Hep G2 RNA contains a mix of IR-A and IR-B cDNA, both of which would be amplified during PCR.

Using Hep G2 cDNA as template, insulin receptor PCR was executed using the Finnzyme Phusion High-Fidelity PCR kit (Thermo Fischer) and thermocycling conditions presented in Table 4-3. Phusion DNA polymerase is a Pyrococcus-like enzyme which has been shown to have 50 times more fidelity than the more traditional Thermus aquaticus DNA polymerase. As such it was reasoned it would be a superior choice for cloning. Each 50 reaction contained 5µL cDNA, 10µL of 5x High Fidelity Phusion buffer, 1µL of 10mM dNTP (200µM final concentration), 2.5µL of 20µM forward and reverse primer (0.5µM final concentration), 2.5µL DMSO (5%) and 0.5µL of Phusion DNA polymerase (0.02U/µL).

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95</td>
<td>30 sec</td>
<td>30</td>
</tr>
<tr>
<td>Annealing/Extension</td>
<td>72</td>
<td>2.30 min</td>
<td></td>
</tr>
<tr>
<td>Final extension</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Hold</td>
<td>4</td>
<td>∞</td>
<td>1</td>
</tr>
</tbody>
</table>

The optimal annealing temperature for Phusion Hot Start DNA polymerase differs significantly from Taq-based polymerases. Consequently, the Tₘ for the primer set described in Figure 4.2-3 was recalculated using the Tₘ calculator available on the Finnzyme’s website (www.finnzyme.com)
The $T_m$ was estimated to be 88ºC and 78ºC for the forward and reverse primer respectively. Because the annealing temperature for both the primers exceeded 72ºC, a two step protocol where the annealing and extension steps are combined was used.

4.2.5.6 OriGene IR-A expression vector

pCMV6-XL4 vector containing IR-A cDNA clone was used to overexpress IR-A isoform (OriGene Technologies) (Figure 4.2-4). The pCMV6-XL4 IR-A clone was derived from a single *E. coli* colony and arrived as a transfection ready plasmid. It was received as 10µg of lyophilized plasmid in a 2-D Matrix tube. Plasmid was reconstituted by adding 100µL of sterile ultra pure H$_2$O giving a final concentration of 100ng/µL. Subcloning Efficiency DH5α (*E. coli*) were transformed with 5µL of IR-A plasmid. It was then propagated and extracted as described previously.

![Map of pCMV6-XL4 vector containing human IR-A clone.](image)

4.2.5.7 Plasmid transfection

IR-A and IR-B plasmids were transfected into Hep G2 cells using branched Polyethylenimine (PEI) (Sigma Aldrich). PEI is a stable cationic polymer with ethylenimine motifs giving it a positively charged backbone. This allows it to bind negatively charged DNA which forms the PEI-DNA complex. This complex can bind to the cell surface and is internalised via endosomal vesicles into the cell cytoplasm.
Stock solutions of PEI were prepared by diluting 1mg of branched PEI in 1mL of sterile RNAase/DNAase free H2O. This solution was then neutralized with HCl and sterilised by filtration through a 0.22µM filter. Aliquotes were stored at -80°C.

Transfection complexes were prepared in sterile RNAase/DNAase eppendorf tubes in master mix format for each plasmid and then added to a 24-well TC-Treated plate (Labcon). The volumes used in the following method description are in per well format. The transfection complex volume for each well was 100µL. Opti-MEM (Life Technologies) was used to dilute both the plasmid and PEI (Sigma Aldrich). IR-A or IR-B (800µg) was diluted in 50µL of Opti-MEM and mixed gently. 2µL of PEI was diluted in 50µL of Opti-MEM and combined with the plasmid dilution. The transfection complex was incubated at room temperature for 20 minutes. During this incubation, Hep G2 cells were trypsinized and centrifuged at 1100 g for 5 minutes. The cell pellet was re-suspended in DMEM without antibiotic/antimycotic and counted in a Neubauer Counting Chamber. The cell suspension was diluted to 100 cells/µL. Transfection complex was added to the bottom of each well after which 500µL of cell suspension was added to each well for a final concentration of 15 x 10⁴ cells/well. Cells were incubated at 37°C and 5% CO₂ for 24 hours after which media was refreshed. Experimental manipulation was conducted 48 hours after transfection.

4.2.6 Insulin treatment of Hep G2 cells

Cells were seeded at 5 x 10⁴/well in a 24-well TC-Treated plate (Labcon) and allowed to attach for 24 hours. Following the 24 hours incubation, insulin (Life Technologies) was added to the medium of four wells for a final concentration of 100nM. The insulin was replenished every 48 hours for 6 days at which point both total RNA and protein was extracted. A further four wells without insulin treatments were used as controls. Total RNA was converted to cDNA as described previously. RT-qPCR was performed using Custom Design TaqMan assays for IR-A and IR-B isoform. Eukaryotic 18s was used as the reference gene. Insulin Receptor protein abundance was assayed by western blot using Anti-Insulin Receptor β-subunit antibody (Clone CT-3 1:500, Millipore). Pan-actin (Clone C4 1:10000 Millipore) was used as a loading control. Relative quantification of protein abundance was done as described previously.
4.2.7 Insulin-induced AKT phosphorylation in cells overexpressing IR-A or IR-B

Experiments were done in triplicate on 3 different days using Hep G2 cells overexpressing IR-A or IR-B isoform and cells transfected with empty pcDNA3.1 vector (control). Cells were serum starved 24 hours prior to experimental manipulation. Cells were stimulated with 100nM insulin for 5 minutes and lysed directly in the well as described previously. Cells not treated with insulin (baseline) were run in parallel. Following 10% SDS-PAGE resolution and transfer to nitrocellulose, membranes were blocked with 5% skim milk powder in TBS-T at 4°C overnight.

Blots were probed with primary antibody diluted in TBS-T for 2 hours at room temperature. Phosphorylated AKT (p-AKT) was assayed using p-Akt1 (Clone 11E6, 1:800, Santa Cruz), a monoclonal antibody corresponding to the phosphorylated Serine 473 residue of human AKT. Anti-Insulin Receptor β-subunit antibody (Clone CT-3, 1:500, Millipore) and Pan-actin (Clone C4, 1:10000, Millipore) were used to confirm overexpression and as a loading control respectively. Blots were stripped as described previously and re-probed for total AKT (t- AKT) using a polyclonal AKT antibody (Clone H-136, 1:2000, Santa Cruz). Relative quantification of phosphorylated AKT protein was calculated by normalizing the intensity of the p-AKT band to the intensity of the t- AKT band.

4.2.8 Insulin-induced inhibition of PCK1 transcription in cells overexpressing IR-A or IR-B

Experiments were done in triplicate on two different days using Hep G2 cells overexpressing IR-A or IR-B isoform and cells transfected with empty pcDNA3.1 vector (control). Cells were serum starved 24 hours prior to stimulation with 1µM insulin for 12 hours. Cells not treated with insulin (baseline) were run in parallel. RNA extraction, cDNA synthesis and RT-qPCR were performed as described previously. No-reverse transcriptase controls were run to control for genomic DNA contamination.
PCK1 TaqMan Gene Expression Assay onDemand (Hs00159918_m1, Applied Biosystems) was used to assay for PCK1 mRNA levels. Eukaryotic 18s was used as a reference gene. Gene expression was presented as fold change according to the $2^{-\Delta\Delta Ct}$ method. Gene expression is also presented as $2^{-\Delta Ct}$ which allowed for visualisation of relative amounts of gene expression as individual data points.

4.2.9 Statistical analysis

Statistical analysis was performed on $2^{-\Delta Ct}$ RT-qPCR data. Consequently gene expression data was expressed as both $2^{-\Delta Ct}$ in graphical form, whereas the fold change for significant differences was reported in text. Statistical analysis of protein abundance was performed on the value generated from normalisation of band intensity to the loading control.

Normal distribution of all groups to be compared was tested with D’Agostino-Pearson test. Non-normally distributed data was log transformed to comply with parametric test assumptions. Pre-post comparisons were carried out using a paired t-test. Student’s t-test was used to test significance between two groups. One way ANOVA was used to test significance between multiple groups. The Pearson product-moment correlation coefficient was used to test the strength and significance of association between variables. An alpha level of 0.05 was set as the significance threshold. All analysis was performed using Minitab15. Graphical visualization was performed using GraphPad Prism 5.
4.3 Results

4.3.1 Liver insulin receptor protein abundance in individuals with type 2 diabetes

Liver IR protein was analysed in individuals who had normal glucose tolerance (NGT group) and type 2 diabetes mellitus (T2DM group) as described previously. IR relative abundance was log transformed prior to analysis with the student’s t-test. The T2DM group had a lower mean IR protein abundance in comparison to the NGT group (Figure 4.3-1A), which was statistically significant (p=0.02). Consequently, in this study, individuals with type 2 diabetes had a -1.3 (95% CI, -1.6 to -1.1) fold lower abundance of IR at the time of RYGB surgery than individuals with normal glucose tolerance. Notably, at the time of RYGB surgery, the T2DM group had mean fasting plasma insulin of 196 pmol/l, whereas the NGT group had mean insulin of 109 pmol/l.

Figure 4.3-1. (A) Mean ± SE IR protein abundance in liver tissue of morbidly obese individuals at the time of RYGB surgery. NGT; Normal glucose tolerance (n=17), T2DM; Type 2 diabetes (n=26). (B) Corresponding western blot for individuals that did not have a second liver biopsy. To avoid gel artefacts all samples to be compared were loaded onto the same E-PAGE™ gel, while spaces delineate different gels. Samples with an * were not included in analysis. For complete western blot film see Appendix.
Consequently, IR protein abundance was associated with circulating insulin and HOMA-IR (Figure 4.3-2). Data from the NGT and T2DM groups was combined and log transformed prior to calculation of a Pearson product-moment correlation. Insulin Receptor abundance negatively correlated with fasting plasma insulin concentration (Figure 4.3-2A) and HOMA-IR index (Figure 4.3-2B).

Figure 4.3-2. (A) Pearson product-moment correlation of liver IR protein relative abundance (log) vs fasting plasma insulin concentration (log). (B) Pearson product-moment correlation of liver IR protein relative abundance (log) vs HOMA-IR (log).

4.3.2 Liver insulin receptor protein abundance after RYGB surgery

Figure 4.3-3A presents the mean relative IR protein abundance at the time of RYGB surgery and approximately 16 months after. Both the sNGT and sT2DM group tend to have an increase in IR protein after RYGB surgery, although the difference does not reach statistical significance for the sNGT (p=0.183, paired t-test) or the sT2DM group (p=0.08, paired t-test). However, regrouping the data into pre and post RYGB groups produces a statistically significant (p=0.01, paired t-test) difference, most likely due to the increased sample size. Consequently, there was a significant 1.4 fold (95% CI, 1.1 to 1.8) increase in IR content after RYGB surgery. Increased IR content was associated with a decrease in mean circulating insulin concentrations in both the sNGT (80 to 27 pmol/l) and sT2DM (219 to 48 pmol/l) groups.
Figure 4.3-3. (A) Mean ± SE IR protein abundance in liver tissue of morbidly obese individuals before and after RYGB surgery (● sNGT, n = 6; ○ sT2DM, n = 7). Mean IR abundance increased significantly after RYGB surgery (paired t-test). (B) Corresponding western blot for IR for individuals that had a second liver biopsy. To avoid gel artefacts all samples to be compared were loaded onto the same E-PAGE™ gel, while spaces delineate different gels. For complete western blot film see Appendix.

4.3.3 IR-B:A mRNA ratio in individuals with type 2 diabetes

Liver IR-A and IR-B mRNA expression was assayed at the time of RYGB surgery. Relative expression levels of IR-A and IR-B mRNA are presented as $2^{-\Delta Ct}$ in Figure 4.3-4B and C. Neither IR-A or IR-B transcript showed significant difference among NGT and T2DM groups, although IR-A expression tended to be higher in the T2DM group (Figure 4.3-4B). The ratio of IR-B:A was calculated by dividing the IR-B $2^{-\Delta Ct}$ values by the IR-A $2^{-\Delta Ct}$ values. The mean IR-B:A ratio in the T2DM group (5.2) was significantly lower than the mean IR-B:A ratio in the NGT group (6.6), (p = 0.04, t-test).
4.3.4 IR-B:A mRNA ratio before and after RYGB surgery

Expression levels of IR-A and B were also analysed in individuals who had normal glucose tolerance (sNGT) or type 2 diabetes (sT2DM) before and after RYGB surgery. The fold change was calculated using the $2^{-\Delta\Delta C_t}$ method by using the pre-RYGB sample as baseline, while the IR-B:A ratio was calculated by dividing the IR-B $2^{\Delta C_t}$ values by the IR-A $2^{\Delta C_t}$ values. Liver IR-B:A isoform ratio increased after remission of insulin resistance and type 2 diabetes as shown in Figure 4.3-5A.
The mean IR-B:A ratio in the sT2DM group increased significantly (p=0.02, paired t-test) from 5.4 (95% CI, 4.0 to 6.7) to 8.6 (95% CI, 7.1 to 10.1) after RYGB surgery, returning to levels seen in the sNGT group. No significant change was seen in the mean IR-B:A ratio after RYGB surgery in the sNGT group.

The change in IR-B:A ratio in individuals who experienced remission of type 2 diabetes was due to a statistically significant (p=0.01, paired t-test) decrease in IR-A mRNA expression following surgery/remission, rather than an increase in IR-B mRNA expression (Figure 4.3-5B and C). IR-A mRNA expression decreased -1.5 fold (95% CI, -2.2 to -1.1) in the sT2DM group after RYGB surgery, whereas this was not seen in the sNGT group.

Figure 4.3-5. IR isoform relative expression in liver tissue of morbidly obese individuals before and after RYGB surgery (● sNGT, n = 8; ○ sT2DM, n = 8). (A) Mean + SE IR-B:A mRNA expression ratio calculated by IR-B 2^(-ΔCt)/IR-A 2^(-ΔCt). The mean IR-B:A ratio had a statistically significant increase after RYGB surgery in the sT2DM group (paired t-test). (B) Mean IR-B mRNA expression and (C) mean IR-A mRNA expression + SE presented as 2^(-ΔCt) normalised to 18S. Mean IR-A mRNA expression had a statistically significant decrease after RYGB surgery in the sT2DM group (paired t-test).
Consequently, the reduced IR-A mRNA expression only occurred in individuals who had remission of type 2 diabetes (general liner model, p=0.04) with expression retuning to levels seen in individuals with normal glucose tolerance after RYGB surgery. There was no statistically significant change in IR-B mRNA expression after RYGB surgery in either group. Because IR can form hybrid heterodimers with IGF-1R, the expression level of IGF-1R was also assayed. There was no statistically significant changes in IGF-1R expression levels after RYGB in either group with the sNGT group having a mean 1.3 fold change (95% CI, -1.1 to 1.7) and sT2DM group having a mean 1.0 fold change (95% CI, -1.4 to 1.4).

4.3.5 IR abundance and relative isoform expression in Hep G2 cells treated with supraphysiological levels of insulin

The purpose of this experiment was to investigate how chronic exposure of liver cells to high concentrations of insulin affects insulin receptor isoform expression. Hep G2 cells were treated with 100nM insulin over 6 days. Cells not treated with insulin were grown simultaneously and used as controls. IR protein abundance and isoform expression is shown in Figure 4.3-6.

Figure 4.3-6. Mean + SE Insulin Receptor protein abundance (A) and isoform expression presented as $2^{-\Delta Ct}$ normalised to 18S (B) in Hep G2 cells treated with insulin (n=4).
Cells treated with 100nM insulin had a significantly lower abundance of IR in comparison to control cells (Figure 4.3-6A) \((p=0.04)\). However, there was no difference in insulin IR-A or IR-B mRNA expression after insulin treatment (Figure 4.3-6B). Consequently, insulin treatment did not change the IR-B:A ratio. Notably, unlike normal human liver cells, Hep G2 cells have 2 fold higher expression of IR-A mRNA.

### 4.3.6 Functional studies of IR isoform specific insulin signalling

Because of the change in IR-B:A isoform expression after remission of diabetes, we wanted to evaluate the functional consequences of signalling through the IR-A isoform in a human liver cell line. Investigation of isoform specific insulin signalling was first attempted with siRNA-mediated knockdown of IR-A or IR-B expression. Hep G2 cells were either transfected with siRNA that targeted the exon 10/12 boundary (IR-A) or siRNA that targeted exon 11 (IR-B). None of the custom design siRNA targeting specific IR isoforms caused efficient or specific knockdown. Although IR-A siRNA s244179 caused complete knockdown of insulin receptor protein (Figure 4.3-7A), it was not specific to the IR-A isoform as it also decreased IR-B expression (Figure 4.3-7B). Neither of the IR-B siRNA (s239120 and s239121) caused significant knockdown of insulin receptor protein (Figure 4.3-7A).

![Figure 4.3-7](image)

Figure 4.3-7. siRNA-mediated knockdown of insulin receptor A and B isoform. (A) Insulin receptor relative abundance and (B) IR-B and IR-A mRNA % knockdown in Hep G2 cells transfected with siRNA \((n=3)\).
Although IR-B siRNA s239121 did not downregulate IR-B mRNA expression, IR-B siRNA s239120 did achieve inefficient non-specific knockdown of both isoforms (Figure 4.3-7B). siRNA targeting a homologues region of the IR isoforms (s7477 and s7479) were added for comparison.

4.3.7 Overexpression of IR-B and IR-A in Hep G2 cells

Because siRNA-mediated knockdown of IR-A or IR-B isoform was not feasible, mammalian vectors were used to over express the IR isoforms. The pcDNA 3.1 plasmid was used to over express IR-B isoform. Initially, cloning of IR-A transcript into the pcDNA 3.1 plasmid was going to be used for overexpressing IR-A. However there were some technical difficulties.

Amplification of non complex templates such as plasmid DNA was only possible after extensive optimization and use of both the Touchdown technique and DMSO as an additive. Figure 4.3-8A shows a DNA agarose gel of Touchdown-PCR product using IR specific primers and linear pcDNA3.1 IR-B plasmid as template. There is a species migrating at ~4100bp representing the IR and confirming the presence of IR-B transcript.

![Figure 4.3-8](image.png)

**Figure 4.3-8.** (A) Insulin receptor PCR using linear pcDNA3.1 IR-B showing a prominent species at ~4100bp and a non specific product at ~150bp. (B) HindIII and XbaI digestion of pcDNA3.1 IR-B. (C) DMSO and MgCl₂ gradient of insulin receptor PCR using Taq man enzymes and Hep G2 cDNA. (D) Successful insulin receptor PCR using Phusion enzyme and Hep G2 cDNA.
Furthermore, cutting the plasmid with HindIII and XbaI released the insert which migrated at the size (~4100bp) expected of the IR (Figure 4.3-8B). However, there is also a smaller species at approximately 150bp that was amplified during IR PCR. This was most likely non-specific priming due to the high GC content of the forward primer (first 50bp of the IR sequence has >80% GC content) and a sequence of five G nucleotides present in 3’-end of the IR transcript. This mis-priming produced a 150 base pair size fragment, which was visible in all PCR reactions using the IR specific primers.

Because of the complex and GC rich nature of the transcript, amplifying the IR transcript with cDNA transcribed from Hep G2 RNA using traditional Taq enzyme was not possible. Varying reaction components such as MgCl₂ and DMSO concentration did not aid in amplifying the IR transcript from complex templates such as cDNA (Figure 4.3-8C). Amplification of IR from cDNA was only possible using proof-reading enzymes such as the Phusion DNA polymerase (Figure 4.3-8D). However, non specific priming was still interfering with the PCR reaction leading to a decreased amount of IR transcript yield. Digestion and ligation reactions were attempted but because of the relatively low yield of insulin receptor PCR product further optimization was necessary.

**4.3.8 Confirmation of isoform specific over expression**

Due to time and budgetary constraints relating to laboratory access, a transfection ready plasmid carrying the IR-A cDNA was purchased from OriGene. This plasmid was used to overexpress IR-A in Hep G2 cells.

Figure 4.3-9 is a representative gel of IR PCR fragments amplified from cDNA reverse transcribed from Hep G2 cells transfected with the pCMV6-XL4 IR-A (Hep G2 IR-A) or pcDNA3.1 IR-B vector (Hep G2 IR-B). To confirm overexpression, PCR was only allowed to proceed for 20 cycles. As expected no species are visible in the Hep G2 Empty Vector (control) lane. However, in the Hep G2 IR-A lane, a fast migrating species at ~60bp is present and is representative of the IR-A fragment as it lacks exon 11. The slower migrating species visible in the Hep G2 IR-B lane at ~96bp is representative of IR-B as it contains exon 11 (36bp).
Figure 4.3-9. Insulin receptor exon 10-12 fragment PCR in Hep G2 cells overexpressing IR-A or IR-B isoform. Eukaryotic 18S was used as a loading control. PCR was run for 20 cycles only.

4.3.9 AKT phosphorylation in Hep G2 cells overexpressing IR-A or IR-B

Insulin-induced metabolic signalling was studied in Hep G2 cells transfected with either IR-A (Hep G2 IR-A) or IR-B (Hep G2 IR-B) cDNA containing plasmid. Cells transfected with empty vector were used as control (Hep G2 EV).

Figure 4.3-10A is a representative western blot of nine that were used to quantify activation of AKT and confirm receptor overexpression. All cells were starved overnight prior to insulin stimulation; empty vector was used as control (Hep G2 EV). Insulin-induced metabolic signalling was investigated by stimulating cells with 100nM insulin for 5 minutes and assaying levels of Akt-Ser$^{473}$ phosphorylation. Total AKT abundance was used to normalise p-AKT abundance. Overexpression of insulin receptor is evident in Hep G2 IR-A and Hep G2 IR-B cells. Actin was used as a loading control.
As shown in Figure 4.3-10A, overexpressing IR-B, but not IR-A, significantly increased AKT phosphorylation compared to cells transfected with empty vector (p=0.01, ANOVA).

Figure 4.3-10. AKT phosphorylation in cells overexpressing IR isoform A or B. (A) Mean + SE AKT phosphorylation of 9 biological replicates over three days. (B) Representative western blot of AKT phosphorylation in HepG2 cells overexpressing IR-A (Hep G2 IR-A) or IR-B (Hep G2 IR-B) after treatment with insulin. For complete western blot film see Appendix.
4.3.10 Insulin-induced PCK1 mRNA expression in Hep G2 cells overexpressing IR-A or IR-B

Insulin regulates glucose homeostasis partially through inhibiting transcription of PCK1 mRNA that codes for PEPCK, a key enzyme in the gluconeogenesis pathway. Consequently, the ability of the individual IR isoforms to regulate PCK1 expression was investigated. Figure 4.3-11 shows PCK1 mRNA expression after insulin treatment in cells overexpressing each isoform. All cells were starved overnight prior to insulin stimulation; empty vector was used as control. Cells were treated with 1µM insulin for 12 hours after which PCK1 mRNA expression was estimated by RT-qPCR.

![Graph showing PCK1 mRNA expression](image)

Figure 4.3-11. Mean + SE PCK1 mRNA expression presented as $2^{-\Delta\Delta C_T}$ normalised to 18S in HepG2 cells overexpressing IR-A or IR-B after treatment with insulin (n=6).

Cells transfected with empty vector had a mean -1.7 fold (95% CI, -2.7 to -1.1) reduction in PCK1 mRNA expression after insulin treatment. Similarly, Hep G2 cells overexpressing IR-B isoform had a mean -2.6 fold (95% CI, -4.6 to -1.5) reduction in PCK1 mRNA expression after treatment with insulin. However, Hep G2 cells overexpressing IR-A tended to have a slight increase in PCK1 mRNA expression (-1.1 fold, 95% CI -1.4 to 1.6) which was not statistically significant (p=0.76, students t-test). Collectively these data suggest that high levels of IR-A expression may have a detrimental impact on insulin-induced inhibition of gluconeogenesis.
4.4 Discussion

The purpose of this study was to investigate whether IR isoform ratios change in individuals who lose insulin resistance through RYGB surgery. IR protein abundance and isoform mRNA expression was assayed in liver of individuals with normal glucose tolerance or type 2 diabetes. Regardless of the status of glucose tolerance, all participants studied before and after RYGB surgery had an improvement in insulin sensitivity and a decrease in fasting plasma insulin (Table 2-3). Individuals with type 2 diabetes had a normalisation of fasting plasma glucose and HbA1c levels indicating a return to normoglycemia.

At the time of RYGB surgery, individuals with type 2 diabetes had lower liver IR protein abundance in comparison to individuals with normal glucose tolerance (Figure 4.3-1A). Downregulation of IR by insulin has previously been demonstrated in vitro.\textsuperscript{153, 154, 156, 157} Considering that individuals with type 2 diabetes, on average, had significantly higher fasting plasma insulin levels, it is likely that the higher concentrations of insulin induced degradation of the IR.\textsuperscript{155, 530} Not surprisingly, IR abundance negatively correlated with fasting plasma insulin concentration ($r = -0.51$, $P<0.001$). Furthermore, liver IR protein abundance increased after RYGB surgery (Figure 4.3-3A). This increase was associated with a decrease in fasting plasma insulin concentration and has previously been documented in skeletal muscle of individuals who have undergone gastric bypass surgery.\textsuperscript{412} The molecular mechanisms that regulate insulin-stimulated degradation of the IR and its consequent influence on insulin signalling are still relatively unknown. However, evidence suggests the adaptor protein Grb10 mediates entry of the IR into the proteasomal degradation pathway.\textsuperscript{157}

One of the key findings in this study was the change in IR-B:A isoform ratio after RYGB surgery (Figure 4.3-5). In normal human liver, IR-B expression predominates.\textsuperscript{366, 424} The ratio of IR-B to IR-A has previously been reported to be 9.8 in normal lean individuals and 6.9 in obese individuals with and without type 2 diabetes.\textsuperscript{366} However in this study group, obese individuals with type 2 diabetes had a significantly lower IR-B:A ratio (5.2) than obese individuals without type 2 diabetes (6.6). Furthermore, the IR-B:A ratio increased from 5.4 to 8.6 only in those
individuals who had a remission of type 2 diabetes, returning to levels seen in the normal glucose tolerant group before RYGB (IR-B:A ratio 8.5).

The change in IR-B:A ratio after RYGB surgery was primarily driven by reduced expression of IR-A rather than increased expression of IR-B isoform. IR-A mRNA expression had a 50% decrease in individuals who had normalisation of glucose homeostasis and a dramatic improvement in insulin sensitivity (Figure 4.3-5), whereas there was no statistically significant changes in IR-B or IGF-IR expression.

In this study, IR-A expression was associated with severe hyperinsulinemia and fasting hyperglycaemia. Increased IR-A expression has previously been documented in skeletal muscle of a markedly insulin-resistant individual with type 2 diabetes. Similarly, individuals with myotonic dystrophy type 1, a disease characterized by severe insulin resistance, also have increased expression of IR-A mRNA isoform. The aberrant insulin receptor ratio is not seen in other myopathies suggesting it may be specific to disorders that are associated with hyperinsulinemia and impaired regulation of glucose homeostasis. Furthermore, IR-A has been established as a predominantly mitogenic receptor and has been found to be overexpressed in certain cancers. An increasing awareness of the link between hyperinsulinemia and carcinogenesis provides circumstantial evidence for a relationship between hyperinsulinemia and increased IR-A expression.

Although it appears insulin has a role in regulating IR isoform splicing, current evidence in the literature is inconclusive. Treating Hep G2 cells with high concentrations of insulin downregulated IR abundance, but there was no significant change in the isoform expression ratio (Figure 4.3-6). Furthermore, because IR-A has been found to have a role in hepatocyte glucose uptake by forming IR-A/GLUT2 complexes, it is possible hyperglycaemic conditions may induce increased IR-A expression. Even though the splicing molecules that regulate alternative IR splicing have been established, the factors that drive their expression are still unclear. It appears that alternative IR splicing involves both hormonal and metabolic factors.
Current literature and data presented here suggests there may be a relationship between conditions characterized with severe hyperinsulinemia and increased IR-A mRNA expression, which may have consequences for insulin signalling.

4.4.1 Changes in IR isoform expression affect insulin signalling in Hep G2 cells

IR-B and A isoform have previously been identified as regulators of metabolic and mitogenic signalling respectively.534 Because of this and the observed change in IR isoform ratio after improvement in insulin sensitivity and remission of diabetes, we wanted to characterise the functional consequences of signalling through the IR-A. We used Hep G2 cells as a human liver cell model to investigate what roles individual IR isoforms have on insulin signalling relating to glucose homeostasis. Although siRNA were initially used to facilitate this, there were some technical difficulties. Due to the highly homologous nature of the IR isoforms, the only targetable region of the mRNA sequence that differed between the two isoforms was the 36 bp exon 11. This significantly reduced the siRNA sequence permutations and resulted in non-specific knockdown of individual isoforms (Figure 4.3-7). Consequently, plasmids containing either the IR-A or IR-B transcript were used to overexpress each isoform in Hep G2 cells after which insulin-mediated metabolic signalling was investigated.

Insulin transmits metabolic signalling through activation of the IR tyrosine kinase. In turn the activated kinase phosphorylates IRS proteins that interact with PI 3-kinase resulting in activation of AKT. This is critical for the metabolic effects of insulin which includes regulation of glycogen synthesis and gluconeogenesis.127 Hep G2 cells overexpressing IR-B had 50% more AKT phosphorylation than control cells after insulin treatment. However, Hep G2 cells overexpressing IR-A did not have a similar level of increase in AKT phosphorylation (Figure 4.3-10A). This further substantiates the role of IR-B as a metabolic signalling isoform.

Uncontrolled hepatic gluconeogenesis and impaired insulin secretion are characteristics thought to be critical for development of hyperglycaemia.22 Insulin exerts its inhibitory action on gluconeogenesis by inhibiting transcription of $PCK1$ mRNA which codes for an enzyme (PEPCK) that catalyzes one of the first steps in gluconeogenesis.189, 190 Evidence from animal studies suggests that $PCK1$ expression
has a fundamental role in regulating glucose homeostasis and insulin sensitivity.\(^{535,536}\) Data presented in this study suggests that insulin-induced downregulation of \(PCK1\) transcription, and therefore its regulation of gluconeogenesis, is IR isoform specific. In Hep G2 cells overexpressing IR-A, insulin did not downregulate \(PCK1\) expression, while cells overexpressing IR-B had insulin-induced downregulation of \(PCK1\) mRNA expression. Furthermore, cells overexpressing IR-B tended to have a greater downregulation of \(PCK1\) mRNA than control cells (Figure 4.3-11). Taken together, data presented in this study further confirms the role of IR-B as the more metabolically active isoform and suggest that increased expression of IR-A may have detrimental outcomes on insulin-mediated metabolic signalling.

This is in agreement with previous studies that have suggested that increased IR-A expression may contribute to the insulin-resistant state. Skeletal muscle cells from individuals with myotonic dystrophy type 1 had decreased insulin sensitivity and increased IR-A expression.\(^{415}\) In the data presented here, there is elevated IR-A expression in individuals with extreme insulin resistance and type 2 diabetes, which is normalised after RYGB surgery. Increasing IR-A expression not only skews the IR-B:A ratio towards the less metabolically active isoform, but also may increase the formation of IR-A/IR-B heterodimers which may detract from the metabolic actions of insulin by decreasing the amount of IR-B homodimers.\(^{397}\) Whether this alteration in ratio is the cause of insulin resistance or the result of markedly elevated circulating insulin concentration and its associated metabolic milieu is yet to be determined. Even so, the relevance of the two insulin receptor isoforms in regulating insulin signalling, in particular that which leads to metabolic outcomes, is clearly pointed to by this data.

Regardless of the underlying mechanisms governing remission of type 2 diabetes remission after RYGB surgery, there is an associated increase in liver insulin sensitivity.\(^{448,476}\) In this study, this was associated with normalization of the IR-B:A ratio in the liver of individuals who have had a remission of type 2 diabetes. Data presented here suggests that IR-B rather than IR-A is central to the regulation of glucose metabolism at the liver. Skewing the ratio towards IR-A expression may have a detrimental affect on insulin signalling and presents a potential molecular mechanism which may contribute to the pathogenesis of type 2 diabetes.
CHAPTER FIVE: Changes in global hepatic gene expression after RYGB Surgery
5.1 Introduction

In the previous chapters of this thesis there was a focus on candidate genes involved in the primary binding and turnover of insulin and how they may contribute to the pathogenesis of type 2 diabetes. Type 2 diabetes is a polygenic disease involving a complex interaction between environmental and genetic factors which often presents as a heterogeneous mix of phenotypes. Because of the heterogeneous nature of both the disease and the human population it is unlikely there will be one critical gene that will be responsible for the onset of type 2 diabetes. Consequently, this chapter takes a more systemic approach in investigating the changes to the transcription profile before and after RYGB surgery in the liver, a key metabolic organ.

Microarray gene expression profiling is an excellent tool that allows biologists to observe global changes in gene expression that occur before and after a given stimulus. In conjunction with gene annotation databases, microarray gene analysis provides an opportunity to identify critical pathways involved in disease progression which can be the subject of further study. This approach has been used to great affect in both animal and human studies. Recently, there has been a focus on identifying the changes in gene expression during the progression from insulin resistance to type 2 diabetes. Several animal model studies suggest that genes in pathways regulating inflammation and hepatic lipid metabolism may have a causal role in the development of diabetes.

Genes regulating inflammation have been shown to be decreased in studies using human muscle and liver following improvements in metabolic parameters after RYGB surgery. Furthermore, genes that regulate lipid metabolism are also differentially expressed after RYGB surgery. However, one key distinction these studies did not address was the possible difference between individuals with and without type 2 diabetes. As shown in Chapter 2, RYGB surgery can induce durable remission of type 2 diabetes that is associated with resolution of insulin resistance. In this chapter we examined the differential global hepatic gene expression before and after RYGB surgery (and remission of diabetes) and between individuals with and without type 2 diabetes.
5.1.1 Experimental strategy

- RNA extracted from liver tissue biopsied from the sNGT and sT2DM group was sent to the Research Centre for Genetic Medicine, Children’s Research Hospital for gene microarray assay on the Illumina platform.
- Raw data was received and subjected to microarray expression analysis using BRB ArrayTools software.
- Select genes that were differentially regulated after RYGB surgery or between individuals with and without type 2 diabetes were further confirmed with TLDA RT-qPCR.
- Analysis of the confirmed genes in the sNGT and sT2DM groups allowed us to control for variable such as weight loss in order to identify genes most likely affected by improvements in insulin resistance or remission of type 2 diabetes.
5.2 Research Design and Methods

The following methods pertain to microarray analysis and confirmatory PCR of liver gene expression in individuals who had normal glucose tolerance (sNGT group) or type 2 diabetes (sT2DM groups) before and after RYGB surgery or remission of diabetes. For detailed description of study cohort refer Chapter 2. Microarray data on one sample from the sT2DM group was not available.

5.2.1 RNAstable transport to Children’s Research Hospital

Previously extracted RNA was sent to Research Centre for Genetic Medicine, Children’s Research Hospital, Washington DC. Microarray gene analysis was conducted by staff at the Core facility using Illumina Bead Microarray analysis. Total RNA was extracted as described in Chapter 2 Research Design and Methods and was sent using RNAstable technology (Biomatrica). RNA was preserved using the principle of anhydrobiosis which enables long term RNA storage. Total RNA (5µg) in DEPC-treated water was aliquoted into a 96-well RNAstable plate. The plate was left to dry overnight in a laminar flow hood. After drying, the plate was sealed with an aluminium foil seal and placed in a moisture barrier foil bag. To recover RNA, 10µL of DEPC-treated water was added and incubated for 15 minutes at room temperature. RNA was then ready for downstream applications.

5.2.2 cRNA synthesis

RNA amplification has become the standard method for preparing RNA for microarray analysis as it facilitates gene expression analysis with minimal sample amount. Amplification of RNA prior to microarray analysis increases both the sensitivity and quality of the data. The Illumina® TotalPrep™ -96 RNA Amplification Kit (Ambion) was used to amplify and synthesize biotinylated cRNA for gene expression analysis on an Illumina platform. This method is based on the RNA amplification protocol as described by Van Gelder et al. (1990). The reaction consists of an oligo (dT) primer bearing a T7 promoter using ArrayScript™, a reverse transcription enzyme specifically design to produce higher yields of first strand cDNA.
than more traditional enzymes. The cDNA then undergoes second strand synthesis after which it is purified and used as a template for in vitro transcription with the MEGAscript ® T7 RNA polymerase and biotin-UTP. This generates thousands of biotinylated antisense RNA copies of mRNA in a sample.

Aliquots of 150ng of high-quality total RNA from each sample was used for cRNA synthesis as per the manufacturer instructions. The concentration of each RNA sample was determined by NanoDrop® Spectrophotometer ND-1000 (NanoDrop Technologies) and the quality of RNA samples was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies Inc). Briefly, RNA was converted to cDNA with Reverse Transcription Master Mix for 2 hours at 42°C. Following this Second Strand Master Mix was added to each sample and incubated at 16°C for 2 hours. The double stranded cDNA was purified via a cDNA Filter Cartridge and the eluted cDNA was used in an in vitro transcription reaction along with IVT Master Mix to synthesize biotinylated cRNA. The cRNA was then purified via a cRNA Filter Cartridge and the resulting product was used for microarray analysis.

5.2.3 Illumina microarray

RNA expression profiling was performed using Illumina® Gene Expression BeadChip Array technology and the Direct Hybridization Assay system (Illumina Inc., San Diego, CA).

Illumina microarray technology is based on arrays of randomly assembled glass silica beads that are 3 microns in diameter and spaced ~5.7 microns apart. Each bead has ~1 million identical probes (50-mer) covalently attached to the surface through an amine linkage. Different bead types are pooled together to form assay panels while every panel has an average of 30 identical bead types producing a high level of bead redundancy. Because the beads are randomly distributed across the substrate surface they must be decoded to identify the bead type and location.

Decoding is achieved by a highly effective hybridization process (error rate < 1 x 10⁻⁴ per bead) from a 29-mer address sequence attached to each bead. This process also validates the hybridization performance of every bead on every BeadChip ensuring the arrays are of a high quality.
In this experiment, assays were conducted using the Illumina Expression BeadChip format. A BeadChip is a planar silica substrate slide that has uniform wells created by plasma etching. The arrays are arranged in a multiple sample format which reduces sample-to-sample variability. 750ng of biotinylated cRNA was detected by hybridization to the 50-mer probes on the HumanHT-12_v4_BeadChip (Illumina Inc). The HumanHT-12 Expression BeadChip has genome-wide transcriptional coverage of well characterized genes, candidate genes, and splice variants. Each array has more than 47,000 probes, which were designed to cover content from the NCBI RefSeq Release 38 (November 7, 2009) as well as legacy UniGene content (Table 5-1).

Table 5-1. Illumina HumanHT-12_v4_BeadChip expression content

<table>
<thead>
<tr>
<th>Probes</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM</td>
<td>Coding transcript, well established annotation (28,688)</td>
</tr>
<tr>
<td>XM</td>
<td>Coding transcript, provisional annotation (11,121)</td>
</tr>
<tr>
<td>NR</td>
<td>Non-Coding Transcript, well established annotation (1,752)</td>
</tr>
<tr>
<td>XR</td>
<td>Non-Coding Transcript, provisional annotation (2,209)</td>
</tr>
<tr>
<td>Source</td>
<td>RefSeq source release (Human RefSeq Release 38)</td>
</tr>
<tr>
<td>UniGene</td>
<td>Experimentally confirmed mRNA sequences that align to ETS clusters</td>
</tr>
</tbody>
</table>

cRNA hybridization and microarray analysis was done according to the Whole-Genome Gene Expression Direct Hybridization protocol (Illumina Inc). After normalizing, the cRNA was dispensed to the BeadChips which were placed in Hyb Chamber inserts and then into Hyb Chambers. The chambers were incubated in an Illumina Hybridization Oven for 16 hours at 58ºC. After incubation the BeadChips were removed, washed with Wash E1BC buffer and blocked with Block E1 buffer. To detect the signal the BeadChips were treated with Cy3-streptavidin for 10 minutes. After a final wash with Wash E1BC buffer the BeadChips were dried via centrifugation and scanned using the HiScan™SQ Reader. Obtained decoded images were analyzed by GenomeStudio™ Gene Expression Module (Illumina Inc).
5.2.4 GeneomeStudio™ Gene Expression Module data output

Data files were received from the Research Centre for Genetic Medicine, Children’s Research Hospital in Microsoft Excel format and included; Probe ID, Gene Symbol, Average Signal, Detection P-value, Search Key, Illumina Gene key and Chromosome Location and Gene Definition. The Average Signal is the average intensity of the bead type/target in the group while the Detection P-value is a statistical calculation that gives the probability the signal from a given bead type is greater than the average signal from the negative controls. The minimum statistically significant detectable fold change for any given gene is 1.35 fold while a detection p-value less than 0.05 signifies that there is 95% chance the gene expression was accurately detected.

The Excel files were received as: Raw Data.xls, Background Corrected.xls, Background Corrected and Normalized.xls and Normalized not background Corrected.xls. According to Dunning et al. (2008), Illumina local background subtraction is beneficial for detecting differential gene expression. However, background normalization may introduce substantial variability, particularly at low intensities and may also increase the amount of false positives. Differential gene expression analysis was initially carried out using the Background Corrected.xls file; however, because in this instance negative values post background correction caused missing values, the Raw Data.xls file was used for gene expression analysis instead.

5.2.5 Analysis of microarray data with BRB ArrayTools

Gene expression microarray analysis is becoming a standard tool in most Molecular Biology laboratories for hypothesis forming or screening for molecules involved in disease pathology. Valid analysis of gene expression microarray experiments requires substantial knowledge of statistical methods and is often done by experts in the Bioinformatics field. However, recent development of analysis software allows biomedical scientists to use valid and powerful statistical methods to achieve their experimental objectives without the need to learn programming language. Biometric Research Branch (BRB) ArrayTools version 4.3.1 software, developed by Dr. Richard Simon and the BRB-ArrayTools Development team (BRB, National Cancer Institute,
US), was used to filter and execute statistical analysis of the microarray data. BRB ArrayTools contains analytic and visualization tools that are integrated into excel via add-ins and use the R version 3.0.1 (R Core Development Core Team, 2013) environment to facilitate analysis. Although an understanding of the processes behind microarray gene analysis is still required, BRB-Array Tools simplifies the complex and computationally intensive processes that generate significant differential gene expression.

5.2.5.1 Processing, collating and filtering the data

The Raw Data.xls file was converted to a tab delimited file (Raw Data.txt) and imported into BRB ArrayTools using the General Format Importer. The data was filtered using the Re-filter, Normalise and Subset the Data function. The Spot Flag Filter function was used to exclude any Average Probe Signal that did not have a significant detection p-value (<0.05) thus allowing only accurately detected genes to be analysed. In addition, only those genes that were present in > 50% of arrays and had expression that differed $\geq 1.35$ fold from the median in at least 20% of the arrays were used for analysis.

5.2.5.2 Data normalisation

Microarray data was normalised to remove any non-biological variation introduced by differences in sample preparation and the production or processing of arrays. Data was normalised using quantile normalisation (Figure 5.2-1).\textsuperscript{555} This is a complete data normalisation algorithm which combines data from all arrays to form the normalisation relation. Quantile normalisation was chosen over other methods for two reasons. Firstly, as it uses the complete data set to execute normalisation, it is more representative of the actual experiment. And secondly it has been shown to be superior when making pairwise comparisons.\textsuperscript{555}
Figure 5.2-1. Box and whisker plots of expression data from all 30 arrays before and after Quantile Normalisation. Boxes show the 25th and 75th percentile of the distribution of log transformed intensities. The horizontal line in the middle of the box represents the median. The dotted lines represent the minimum and maximum values. The circles represent any outliers that lie outside 1.5 times the interquartile range.
5.2.6 Gene annotation

Genes that passed filtering were annotated using the SOURCE database which was designed particularly for microarray gene analysis.\textsuperscript{556} SOURCE combines many publicly available databases (OMIM, SwisProt, UniGene, GenBank and others) and unifies information on gene function, gene ontology and gene expression data.\textsuperscript{556-560} SOURCE contains gene reports for both characterised and uncharacterised genes. Characterised genes are titled with Human Gene Nomenclature Committee approved conventions,\textsuperscript{561} while uncharacterised genes are named using their UniGene titles.

5.2.7 Class comparison

In this study, differentially expressed genes were identified by using the multivariate permutation test (MPT) in BRB ArrayTools. The MPT is a type of class comparison statistical test which uses supervised methods that stratify specimens by class.

Differential gene expression after RYGB surgery was identified by comparing the pre-RYGB (sNGT and sT2DM combined) vs post-RYGB classes (sNGT and sT2DM combined), pre sNGT vs post sNGT classes and pre sT2DM vs post sT2DM classes.

Differential gene expression between individuals with and without type 2 diabetes was identified by comparing sNGT vs sT2DM classes (pre and post data combined), pre sNGT vs pre sT2DM classes, and post sNGT vs post sT2DM classes. All intensity data was log\textsubscript{2} transformed prior to statistical analysis.

5.2.8 Multivariate Permutation Test and the Random Variance Model

Genes that were differentially expressed were identified using a multivariate permutation test (MPT) with a random variance model (RVM) t-test.\textsuperscript{562-564} The MPT is a statistical test designed to control the number or proportion of false discoveries. It is especially effective for experiments with a small sample size where the assumption of a Gaussian distribution may be false.\textsuperscript{563, 565}
False discoveries are a major issue in statistical analysis of microarray data due to the multiple comparison problem created by testing for statistical significance between thousands of different genes. Although several methods for controlling the number of false discoveries have been developed there are some problems with these approaches. The Bonferroni adjustment to p-values is a popular, simple, and effective technique that controls the number of false positives. However, it is often too conservative for application in microarray studies and may lead to false negative results because of the inherent correlation present in gene expression.

Benjamini and Hochberg developed a less conservative technique that controls the proportion of false positives otherwise termed the false discovery rate (FDR). Although this technique is viable and still widely used it also operates under the assumption of independence, which may not be the case in genes that are co-regulated. Furthermore, these techniques are based of p-values calculated using parametric t/F tests that may not be accurate in the extreme tails of the normal distribution for a study with a small sample size.

The small sample size in this study was partly addressed by use of a random variance model. Standard t/F tests are based on the assumption that the within class variance is different for each gene. Consequently the variance for each gene is estimated separately. However, when the sample size is small this estimation is inaccurate and the statistical power of the t/F test is poor. The RVM t-test permits the sharing of information among probe sets without assuming that all the probe sets have the same variation. Specifically, the RVM t-test assumes that the genes have different variances, but it regards these variances as independent samples from the same distribution. The variances are drawn from a two parameters ($\alpha$ and $\beta$) inverse gamma distribution whose parameters are estimated from the complete set of expression data. In studies where the sample size is small the RVM increases the degrees of freedom, which improves estimation of variances and statistical power for detecting differentially expressed genes.

Using the RVM t-test p-values in a MPT avoids the assumption that the errors have a Gaussian distribution and as such is more accurate. During each permutation in the MPT, the class labels are randomly reassigned and the p-value is recalculated for each probe set.
The generated p-value for each gene is a measure of the extent it appears differentially expressed between the random classes generated by the permutations (a highly significant p-value represents a false positive).\textsuperscript{565} The genes are then ordered by their permutation p-values and a p-value threshold is set. A large number of permutations are made and for each potential p-value threshold the program records the number of genes on the list. The distribution of the genes that have a smaller p-value than the threshold (false positives) can be calculated. The algorithm selects a threshold p-value so that the proportion of false discoveries is no greater than C\% of the time (C denotes the confidence interval).

In this study, a total of 1000 permutations were completed to identify the list of probes sets containing < 5\% false positives at a confidence of 90\%. Differential expression was considered significant at $p \leq 0.001$.

5.2.9 TaqMan® Low Density Array confirmatory RT-qPCR

Confirmatory PCR was carried out on genes that had a fold change of 1.35 fold or higher in any direction and had highly significant p-value ($p =/<0.001$) from class comparison with multivariate permutation test. Genes that were differentially regulated in the sT2DM group after RYGB surgery or were differentially expressed between individuals with and without type 2 diabetes were given priority for confirmation. Twelve TaqMan® Low Density Arrays (TLDAP) were purchased from Applied Biosystems. Each TLDA consisted of 384 wells preloaded with 48 TaqMan® Gene Expression Assays, 3 of which were reference genes (Figure 5.2-2). Each TLDA came in a TaqMan® Array Micro Fluidic Card format which was capable of assaying 8 samples simultaneously.
5.2.9.1 TaqMan® Array Micro Fluidic Card loading, sealing and analysis

First strand cDNA synthesis was carried out as described in Chapter 3 Research Design and Methods. cDNA was divided into 11µL aliquots and stored at -20°C. Samples were transported frozen to the Cancer Genetics laboratory, Department of Biochemistry, University of Otago, Dunedin for analysis on the ABI Prism® 7900HT Sequence Detection System.

Frozen cDNA was thawed on ice and 10µL was added to a microcentrifuge tube containing 40µL nuclease free water and 50µL of 2x TaqMan® Universal PCR Master Mix (Applied Biosystems). The tubes were mixed by gentle vortexing and briefly spun in a mini-centrifuge. The TaqMan® Array Micro Fluidic Card was allowed to reach room temperature (~15min incubation) after which 100µL of the sample-PCR master mix was loaded into the TLDA card reservoirs. The TLDA cards were centrifuged twice in a Heraeus centrifuge using the Heraeus Custom buckets and card holders at 331g for 1 minute at an up/down ramp rate of 9. After centrifugation the TaqMan® probes and primers within the reaction wells were re-suspended.
The wells were sealed using a TaqMan® Array Micro Fluidic Card Sealer which isolates the wells after the sample-PCR master mix is loaded. The TLDA cards were run on an ABI Prism® 7900HT Sequence Detection System (Applied Biosystems). Sequence Detection Software version 2.1 (Applied Biosystems) and Relative Quantitation (RQ) Manager Software were used to calculate a threshold cycle (Ct). Each sample was run in triplicate using 12 TLDA plates.

5.2.9.2 Statistical analysis of TLDA RT-qPCR data

DataAssist™ software was used to compile the Ct data generated by RQ manager. Technical replicates were averaged and a Grubb’s test was used to filter outliers.\textsuperscript{568} Relative Expression Software Tool (REST, 2009) developed by Corbett Research Pty Ltd and Dr Michael W. Pfaffl\textsuperscript{569} was used to estimate and confirm up and down regulation of genes that were found to be significant with microarray analysis.

REST 2009 allows for use of multiple reference genes for normalisation and uses integrated randomization (a similar concept is used in the MPT) to test for statistical significance. The hypothesis test represents the probability that the difference between the sample and control groups is due only to chance.

It randomly permutes class labels up to 10,000 times and counts the number of times the relative expression of the randomly assigned groups is greater than the sample data. In addition, REST 2009 applies bootstrapping techniques that provide 95% confidence intervals for expression ratios, without normality or symmetrical distribution assumptions. Using REST 2009, Ct data was normalised using a normalization factor that was generated by taking the geometric mean of Ct values of the reference genes (\textit{18S}, \textit{ACTB} and \textit{PPIA}). TLDA RT-qPCR data was presented as fold change along with a confidence interval and p-value. Gene expression data was also presented as absolute values relative to the \textit{18S} (\textit{2}^{-\Delta\text{Ct}}).
5.2.10 Analysis of LGALS4 protein abundance using E-PAGE electrophoresis and western blotting

LGALS4 protein abundance was analysed in human liver tissue to confirm the microarray and RT-qPCR results. Briefly, 15 µg of protein per sample was resolved by E-PAGE electrophoresis (Life Technologies) and transferred to Hybond-P PVDF membrane (Amersham). Membranes were blocked with 5% skim milk powder in TBT-T at 4°C overnight and then probed for 2 h at room temperature with Anti-LGALS4 antibody (1:2000, Santa Cruz) Blots were then incubated with alkaline phosphatase-conjugated anti mouse antibody (Life Technologies) for 0.5 h. The chemiluminescent signal was developed using CDP-Star chemiluminesent substrate (Life Technologies) and captured on x-ray film (Kodak). The protein bands on the x-ray film were digitized by the Chemidoc XRS system (Bio Rad) and then analyzed using Quantity One software (Bio Rad). Bands were quantified and expressed as volume quantity; the sum of the intensities of the pixels within a defined volume boundary × pixel area (intensity units × mm²). Local background subtraction was used to facilitate background correction. LGALS4 protein relative abundance was calculated by normalizing the intensity of the LGALS4 band to the intensity of the Actin band.

Statistically significant differences in LGALS4 expression were tested with student’s t-test. Normal distribution of all groups to be compared was tested with D’Agostino-Pearson test. Non-normally distributed data was log transformed to comply with parametric test assumptions. An alpha level of 0.05 was set as the significance threshold.
5.3 Results

5.3.1 Differential gene expression after RYGB surgery

Differential gene expression was analyzed by comparing pre-RYGB gene expression to post-RYGB gene expression in 15 individuals that had second liver biopsies. Three different comparisons were made including: gene expression changes after RYGB surgery in all 15 individuals, gene expression changes in the sNGT group only, and gene expression changes in the sT2DM group only.

Out of the 575 genes that passed filtering criteria, class comparison analysis revealed 71 genes that were significantly differentially expressed (> 1.35 fold) after RYGB surgery. When controlled for disease presence, the sNGT group had only one differentially expressed gene, whereas the sT2DM had 50, 13 of which were only differentially regulated after remission of type 2 diabetes. Table 5-2 presents 84 genes that had a statistically significant change in gene expression organized according to Gene Ontology Biological Process into one of 11 functional categories; glucose metabolism, lipid metabolism, amino acid metabolism, inflammation and immunity, blood coagulation, regulation of transcription, cell growth and differentiation, xenobiotic metabolism, cell structure, transport, and miscellaneous. Only a third of differentially expressed genes were upregulated while the rest were downregulated after RYGB surgery.

5.3.1.1 Glucose metabolism

Eight of the differentially expressed genes were involved in either glucose metabolism or insulin action and most were downregulated. Inhibin, beta E (INHBE), is involved in insulin secretion and was decreased after RYGB surgery. Notably, INHBE, which is a secreted protein, was decreased 2.33 fold in individuals who had a remission of type 2 diabetes. Genes involved in glucose metabolism such as enolase 3 (ENO3), serine dehydratase (SDS) and serine/threonine-protein kinase SIK1 (SIK1) were all decreased after RYGB surgery. Conversely, phosphoenolpyruvate carboxykinase (PCK1) expression was increased after RYGB surgery only in individuals who had a remission of type 2 diabetes.
Table 5-2. Illumina microarray differentially expressed (≥ 1.35 fold) liver genes ~16 months after RYGB surgery (n=15).

<table>
<thead>
<tr>
<th>Illumina ID (ILMN_</th>
<th>Gene (Symbol)</th>
<th>GO Biological Process</th>
<th>Fold Change Post/Pre</th>
<th>P-value</th>
<th>Functional categories</th>
</tr>
</thead>
<tbody>
<tr>
<td>1678904</td>
<td>Enolase 3 (beta, muscle) (ENO3)</td>
<td>Glycolysis, skeletal muscle tissue regeneration</td>
<td>-1.67 (-1.69)*</td>
<td>1.00E-07</td>
<td>Glucose metabolism</td>
</tr>
<tr>
<td>1811114</td>
<td>Serine dehydratase (SDS)</td>
<td>Gluconeogenesis, L-serine metabolism</td>
<td>-1.64 (1.89)#</td>
<td>1.15E-04</td>
<td></td>
</tr>
<tr>
<td>1717639</td>
<td>Serine/threonine-protein kinase SIK1 (SIK1)</td>
<td>regulation of gluconeogenesis/ triglyceride biosynthetic process/mitotic cell cycle</td>
<td>-1.59 (-1.69)*</td>
<td>2.46E-04</td>
<td></td>
</tr>
<tr>
<td>1811767</td>
<td>Inhibin, beta E (INHBE)</td>
<td>Growth, insulin secretion</td>
<td>-1.89 (2.33)*</td>
<td>4.70E-06</td>
<td></td>
</tr>
<tr>
<td>1731948</td>
<td>Phosphoenolpyruvate carboxykinase (PCK1)</td>
<td>Response to insulin stimulus, gluconeogenesis</td>
<td>1.56</td>
<td>4.39E-05</td>
<td></td>
</tr>
<tr>
<td>2053415</td>
<td>Low density lipoprotein receptor (LDLR)</td>
<td>Lipid transport, receptor-mediated endocytosis</td>
<td>-1.72</td>
<td>8.21E-04</td>
<td></td>
</tr>
<tr>
<td>1784871</td>
<td>Fatty acid synthase (FASN)</td>
<td>Fatty acid biosynthesis</td>
<td>-1.69</td>
<td>7.37E-05</td>
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<tr>
<td>2138765</td>
<td>Perilipin-2 (PLIN2)</td>
<td>Cellular lipid metabolic process, lipid storage, long-chain fatty acid transport, development of adipose tissue</td>
<td>-1.52 (-1.69)*</td>
<td>8.15E-05</td>
<td></td>
</tr>
<tr>
<td>2041293</td>
<td>Squalene monoxygenase (SQLE)</td>
<td>Cholesterol biosynthetic process</td>
<td>-1.43</td>
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<td>Lipid metabolism</td>
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<tr>
<td>1670134</td>
<td>Fatty acid desaturase 1 (FADS1)</td>
<td>Unsaturated fatty acid biosynthetic process, response to insulin stimulus</td>
<td>-1.37 (1.56)*</td>
<td>4.48E-04</td>
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<tr>
<td>1683598</td>
<td>Long-chain-fatty-acid--CoA ligase 4 (ACSL4)</td>
<td>Lipid biosynthetic process</td>
<td>-1.47</td>
<td>5.46E-04</td>
<td></td>
</tr>
<tr>
<td>2234956</td>
<td>Leptin receptor (LEPR)</td>
<td>Cholesterol metabolic process, energy reserve metabolic process, cytokine-mediated signalling pathway</td>
<td>1.35</td>
<td>9.00E-07</td>
<td></td>
</tr>
<tr>
<td>2346987</td>
<td>Apolipoprotein(a) (LPA)</td>
<td>Lipid transport, lipid metabolic process, blood circulation</td>
<td>1.46*</td>
<td>1.53E-05</td>
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<tr>
<td>1652037</td>
<td>Uridine phosphorylase 2 (UPP2)</td>
<td>Pyrimidine nucleoside catabolic process</td>
<td>-1.61</td>
<td>9.37E-04</td>
<td>Amino acid metabolism</td>
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<tr>
<td>1757807</td>
<td>Ethanolamine-phosphate phosphohydrolase (AGXT2L1)</td>
<td>Cellular amino acid metabolic process</td>
<td>-1.45</td>
<td>6.33E-05</td>
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157
<table>
<thead>
<tr>
<th>Illumina ID (ILMN_)</th>
<th>Gene (Symbol)</th>
<th>GO Biological Process</th>
<th>Fold Change Post/Pre</th>
<th>P-value</th>
<th>Functional categories</th>
</tr>
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<tr>
<td>2056975</td>
<td>Hypoxanthine-guanine phosphoribosyltransferase (HPRT1)</td>
<td>Guanine salvage, hypoxanthine salvage, purine ribonucleoside salvage, positive regulation of dopamine metabolic process</td>
<td>1.40*</td>
<td>8.00E-07</td>
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</tr>
<tr>
<td>1657862</td>
<td>Adenosylhomocysteinase (AHCY)</td>
<td>Sulfur amino acid metabolic process, circadian sleep wake cycle</td>
<td>1.45*</td>
<td>6.40E-06</td>
<td></td>
</tr>
<tr>
<td>2395451</td>
<td>Argininosuccinate synthase (ASS1)</td>
<td>Arginine biosynthetic process, indirectly involved in control of blood pressure,</td>
<td>1.37*</td>
<td>4.98E-05</td>
<td></td>
</tr>
<tr>
<td>1780575</td>
<td>C-reactive protein (CRP)</td>
<td>Complement activation, classical pathway, negative regulation of lipid storage</td>
<td>-2.56 (-3.33)*</td>
<td>1.19E-05</td>
<td></td>
</tr>
<tr>
<td>1774874</td>
<td>Interleukin-1 receptor antagonist protein (IL1RN)</td>
<td>Negative regulation of interleukin-1-mediated signalling pathway, insulin secretion, immune response</td>
<td>-1.54 (-1.59)*</td>
<td>1.40E-06</td>
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</tr>
<tr>
<td>1687384</td>
<td>Interferon alpha-inducible protein 6 (IF16)</td>
<td>Cytokine-mediated signalling pathway</td>
<td>-1.47*</td>
<td>2.05E-05</td>
<td>Inflammation and immunity</td>
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<td>1778010</td>
<td>Interleukin-21 (IL32)</td>
<td>Immune response</td>
<td>-1.35</td>
<td>2.64E-04</td>
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<td>2357062</td>
<td>Interleukin-1 receptor accessory protein (IL1RAP)</td>
<td>Immune response, inflammatory response</td>
<td>1.63 (1.91)*</td>
<td>2.00E-06</td>
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<td>1744381</td>
<td>Serpine peptidase inhibitor, clade E, member 1 (SERPINE1)</td>
<td>Negative regulation of plasminogen activation*</td>
<td>-2.44 (-3.1)*</td>
<td>3.70E-06</td>
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<td>2411731</td>
<td>Hermansky-Pudlak syndrome 5 protein (HP55)</td>
<td>Blood coagulation</td>
<td>-1.47*</td>
<td>2.38E-05</td>
<td>Blood coagulation</td>
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<td>1745607</td>
<td>Alpha-2-macroglobulin (A2M)</td>
<td>Negative regulation of complement activation/ blood coagulation</td>
<td>1.42*</td>
<td>3.73E-05</td>
<td></td>
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<tr>
<td>1806023</td>
<td>Jun proto-oncogene (JUN)</td>
<td>Negative regulation of DNA binding and transcription, liver development, aging, circadian rhythm, immune response, Regulation of transcription, DNA dependant, multicellular organismal development</td>
<td>-2.38</td>
<td>6.34E-05</td>
<td>Regulation of transcription</td>
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<td>1751607</td>
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*Genes that were differentially expressed when analysis was restricted to those individuals who had a remission of type 2 diabetes (Pre sT2DM versus Post sT2DM, n=7). # Genes that were differentially expressed when analysis was restricted to those individuals who had normal glucose tolerance after RYGB surgery (Pre sNGT versus post sNGT, n=8).
5.3.1.2 Lipid metabolism and amino acid metabolism

Eight genes involved in lipid metabolism were differentially regulated after RYGB surgery. Genes involved in the lipid biosynthetic process such as fatty acid synthase (FASN), fatty acid desaturase 1 (FADS1), squalene monooxygenase (SQLE) and long-chain-fatty-acid--CoA ligase 4 (ACSL4) were all downregulated. Genes involved in lipid transport such as LDL receptor (LDLR) and perilipin-2 (PLIN2) were also downregulated. Conversely, expression of leptin receptor (LEPR) and apolipoprotein(a) (LPA) was increased after surgery.

Five genes involved in amino acid metabolism were differentially regulated after RYGB surgery. Adenosylhomocysteinase (AHCY), a gene involved in the circadian rhythm, and argininosuccinate synthase (ASS1), a gene indirectly involved in control of blood pressure, were both increased only in the sT2DM group. Expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT1), a gene involved in the dopamine metabolic process was also increased after RYGB, whereas the remainder of the genes involved in amino acid metabolism were downregulated.

5.3.1.3 Inflammation, immunity and blood circulation

Five genes involved in inflammation and immunity were differentially regulated after RYGB surgery. Expression of C- reactive protein (CRP) and interleukin-21 (IL32), which are both involved in the immune response, were decreased after RYGB. Notably, CRP expression had a 3.33 fold reduction in the sT2DM group after surgery. There were also significant changes in the IL-1 signalling pathway as expression of interleukin-1 receptor antagonist protein (IL1RN) decreased after RYGB surgery, while expression of interleukin-1 receptor accessory protein (IL1RAP) was increased and was 1.91 fold higher in the sT2DM group after remission of diabetes. Three genes involved in blood coagulation were differentially expressed after RYGB surgery. Expression of serpine peptidase inhibitor, clade E, member 1 (SERPINE1) was decreased after surgery and had a 3.1 fold reduced expression in the sT2DM group. Expression of Hermansky-Pudlak syndrome 5 protein (HPS5) was only decreased in individuals who had a remission of type 2 diabetes while expression of alpha-2-macroglobulin (A2M) was increased.
5.3.1.4 Transcriptional regulation

14 genes involved in regulation of transcription were differentially regulated after RYGB surgery. Jun proto-oncogene (\textit{JUN}) and jun-D (\textit{JUND}), both of which were downregulated after RYGB surgery, regulate transcription of genes involved in a wide range of process including, the immunity response, aging, circadian rhythm and organ development. A number of transcription factors that were differentially regulated were involved in cell growth. Activating transcription factor 3 (\textit{ATF3}), which is involved in positive regulation of cell proliferation and gluconeogenesis was decreased after RYGB surgery. Similarly, FBJ murine osteosarcoma viral oncogene homolog B (\textit{FOSB}) and tribbles homolog 1 (\textit{TRIB1}), which are involved in multicellular organismal development and regulation of MAPK activity respectively, were also decreased after RYGB surgery. Genes involved in the apoptotic process, such as cysteine/serine-rich nuclear protein 1 (\textit{CSRNP1}) and cyclic AMP-dependent transcription factor ATF-5 (\textit{ATF5}) were downregulated. Conversely, zinc finger protein SNAI2 (\textit{SNAI2}), a gene involved in negative regulation of apoptotic process is upregulated in the sT2DM group only.

Multiple genes that are involved in circadian regulation of transcription were differentially regulated after RYGB surgery. Genes such as basic helix-loop-helix family member e40 (\textit{BHLHB2}) and nocturnin (\textit{CCRN4L}) were both downregulated after RYGB surgery. Conversely, aryl hydrocarbon receptor nuclear translocator-like protein 1 (\textit{ARNTL}) was increased after RYGB surgery.

5.3.1.5 Cell growth and differentiation

13 genes involved in regulating cell growth and differentiation were differentially regulated after RYGB surgery. Several of these genes that are involved in negative regulation of cell growth and proliferation as a response to stress were downregulated after RYGB surgery. These included; dual specificity phosphatase 1 (\textit{DUSP1}), tumour protein p53 inducible nuclear protein 1 (\textit{TP53INP1}), DNA damage-inducible protein GADD45 beta (\textit{GADD45B}) and cyclin-dependent kinase inhibitor 1 (\textit{CDKN1A}).
Expression of fibronectin type III domain containing 5 (FNDC5), a gene that regulates differentiation of white fat cells to brown fat cells was also decreased after RYGB surgery. Conversely, expression of dual specificity mitogen-activated protein kinase kinase 1 (MAP2K1), a component of the insulin receptor signalling pathway involved in cell proliferation and differentiation, was increased only in the sT2DM group. Expression of insulin-like growth factor-binding protein 5 (IGFBP5) and insulin-like growth factor-binding protein 2 (IGFBP2) was also increased after RYGB surgery. Both IGFB5 and IGFBP2 are involved in cell growth and regulation of glucose homeostasis through the IGF signalling pathway. Notably, IGFBP2 expression increased 2.82 fold in individuals who had a remission of type 2 diabetes and was the most highly upregulated gene.

5.3.1.6 Xenobiotic metabolism

Eight genes involved in xenobiotic metabolism were differentially regulated after RYGB surgery. Expression of seven of these genes, which are all members of the Metallothionein family, was decreased after RYGB surgery. On the other hand, expression of Cytochrome P450 1A2 (CYP1A2), a gene involved in xenobiotic metabolism and the steroid catabolic process was increased after RYGB surgery.

5.3.1.7 Cellular transport and structure

10 genes involved in regulating cellular transport were differentially regulated after RYGB surgery. Solute carrier family 2 facilitated glucose transporter member 3 (SLC2A3), which is involved in glucose transport, was decreased after RYGB surgery. Similarly, genes involved in intracellular trafficking such as rho-related GTP-binding protein RhoB (RHOB), spectrin beta chain, non-erythrocytic 1 (SPTBN1) and alpha-crystallin A chain (CRYAA) were also downregulated. Conversely, neutral and basic amino acid transport protein rBAT (SLC3A1), a gene involved in regulating amino acid metabolism was increased after RYGB surgery.

Six genes involved in cell structure were differently regulated after RYGB surgery. Genes involved in cell adhesion such as putative uncharacterized protein CNTNAP2 (CNTNAP2) and rho-related GTP-binding protein RhoE (RND3) were downregulated after RYGB surgery.
Microtubule-associated proteins 1A/1B light chain 3A (MAP1LC3A), a gene involved in autophagic vacuole formation was also downregulated, whereas prolyl 4-hydroxylase subunit alpha-1 (P4HA1), a gene involved in collagen fibril formation, was upregulated. Some genes that were differentially regulated had unknown or miscellaneous functions. Expression of Transmembrane protein 45B (TMEM45B) and Transmembrane protein 154 (TMEM154) was decreased after RYGB surgery. Conversely, H19 and Serpin A11 (SERPINA11) both had an increase in expression after RYGB surgery.

5.3.2 Differential gene expression between individuals with normal glucose tolerance or type 2 diabetes

Differential gene expression was analyzed by comparing gene expression in individuals who had normal glucose tolerance to gene expression in individuals who had type 2 diabetes. Three different comparisons were made including, differential gene expression between the sNGT and sT2DM groups (pre and post data combined), differential gene expression between the sNGT and sT2DM group at the time of RYGB surgery (Pre RYGB state), and gene expression between the sNGT and sT2DM group at the time of subsequent surgery (Post RYGB state).

Out of the 575 genes that passed filtering criteria, class comparison analysis revealed 10 genes that were significantly differentially expressed between individuals with and without type diabetes. When controlled for operation time point, two genes were differentially expressed between individuals with and without type 2 diabetes at the initial liver biopsy (Pre-RYGB state) and four genes were differentially expressed at the second liver biopsy (Post-RYGB). Table 5-3 presents the differentially expressed genes organized according to Gene Ontology Biological Process

Expression of genes involved in the immune response such as immunoglobulin lambda-like polypeptide 1 (IGLL1), serum amyloid A-1 protein (SAA1), and immunoglobulin J chain (IGJ) was elevated in individuals who had type 2 diabetes regardless of whether they had RYGB surgery. Similarly, expression of galectin-4 (LGALS4), a gene involved in cell adhesion and carbohydrate binding, and pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2 (PYROXD2), was elevated in individuals who had type 2 diabetes before and after RYGB surgery.
Table 5-3. Illumina microarray differentially expressed genes between the sNGT (n=8) and sT2DM (n=7) groups.

<table>
<thead>
<tr>
<th>IlluminaID (ILMN)</th>
<th>Gene (Symbol)</th>
<th>GO Biological Process</th>
<th>Fold Change sT2DM/sNGT</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>2393765</td>
<td>Immunoglobulin lambda-like polypeptide 1 (IGLL1)</td>
<td>immune response</td>
<td>1.92</td>
<td>5.08E-05</td>
</tr>
<tr>
<td>2304512</td>
<td>Serum amyloid A-1 protein (SAA1)</td>
<td>Negative regulation of inflammatory response, platelet activation, positive regulation of interleukin-1 secretion</td>
<td>1.89</td>
<td>2.23E-04</td>
</tr>
<tr>
<td>1694034</td>
<td>Galectin-4 (LGALS4)</td>
<td>Cell adhesion, carbohydrate binding</td>
<td>1.82 (2)*</td>
<td>1.00E-07</td>
</tr>
<tr>
<td>1684497</td>
<td>Pyridine nucleotide-disulfide oxidoreductase domain-containing protein 2 (C10orf33/PYROXD2)</td>
<td>Oxidoreductase activity</td>
<td>1.66*#</td>
<td>&lt;1e-07</td>
</tr>
<tr>
<td>2105441</td>
<td>Immunoglobulin J chain (IGJ)</td>
<td>Immune response</td>
<td>1.41</td>
<td>3.53E-03</td>
</tr>
<tr>
<td>2188966</td>
<td>Insulin-like growth factor-binding protein complex acid labile subunit (IGFALS)</td>
<td>Signal transduction</td>
<td>-1.4</td>
<td>9.36E-04</td>
</tr>
<tr>
<td>1746220</td>
<td>Dopamine beta-hydroxylase (DBH)</td>
<td>Synaptic transmission, positive regulation of vasoconstriction</td>
<td>-1.48</td>
<td>3.69E-03</td>
</tr>
<tr>
<td>3308138</td>
<td>RNU4-2</td>
<td>spliceosome</td>
<td>-1.49 (1.65)*</td>
<td>5.50E-06</td>
</tr>
<tr>
<td>3309453</td>
<td>RNU4-1</td>
<td>spliceosome</td>
<td>-1.5 (-1.74)*</td>
<td>1.93E-05</td>
</tr>
<tr>
<td>3236653</td>
<td>RNU1-5</td>
<td>spliceosome</td>
<td>-1.54*</td>
<td>2.11E-05</td>
</tr>
<tr>
<td>1729191</td>
<td>Cytochrome P450 2C19 (CYP2C19)</td>
<td>Xenobiotic metabolic process, steroid metabolic process</td>
<td>-1.74</td>
<td>1.13E-04</td>
</tr>
</tbody>
</table>

*Genes that were differentially expressed when analysis was restricted to the Pre-RYGB state (Pre sNGT versus Pre sT2DM). # Genes that were differentially expressed when analysis was restricted to the Post-RYGB state (Post sNGT versus Post sT2DM).
Conversely, expression of insulin-like growth factor-binding protein complex acid labile subunit (IGFALS), a gene involved in signal transduction, and dopamine beta-hydroxylase (DBH), a gene involved in synaptic transmission and vasoconstriction was reduced in individuals who had type 2 diabetes compared to individuals who had normal glucose tolerance. Furthermore, the expression of two genes (RNU4-2, RNU4-1) coding for small nuclear RNA’s that are involved in the spliceosome was lower in individuals who had type 2 diabetes. Expression of RNU1-5 was lower in the sT2DM group only at the second operation.

5.4 Validation of microarray profiles with TLDA RT-qPCR

Of the 95 genes that were found to be significantly differentially regulated by microarray analysis, 45 were chosen for confirmation by Taq Man Low Density Array (TLDA) RT-qPCR. 36 of these genes were differentially regulated after RYGB surgery, whereas nine were differentially regulated between individuals with or without type 2 diabetes. To test the stringency of the microarray analysis three of the 45 genes (RPL10A, CYP1A2, and DBH) that had a MPT p-value between 0.001 and 0.05 in addition to one control gene (HAMP) that had a borderline significant p-value of 0.07 were chosen for further validation with RT-qPCR.

Of the 45 Taq Man probes on the TLDA card, two probes (JUND and IGLL1) failed to produce a detectable signal. The signals from the remaining 43 Taq Man probes were normalized to 3 reference genes (18S, ACTB and PPIA) prior to data analysis. Figure 5.4-1 shows the relative average differential gene expression as determined by microarray or TLDA for 43 genes. For all the confirmed genes there was directional concordance (increased or decreased) between the expression values generated by microarray analysis and TLDA analysis. Microarray gene expression and TLDA RT-qPCR gene expression was highly correlated (Spearman rank, r = 0.93, p<0.0001). In almost all cases the TLDA fold change/difference expression values were greater in magnitude than the fold change determined by Illumina microarray (Figure 5.4-1). Furthermore, differential expression of HAMP, which was not significant with microarray analysis (p=0.07) was confirmed to not be significant with TLDA analysis (p=0.06).
Figure 5.4-1. Comparison of Illumina Microarray and TLDA RT-qPCR fold changes of 43 genes that were found to be differentially expressed. (A) Differentially regulated genes between the sNGT (n=8) and sT2DM (n=7) groups. (B) Differentially expressed genes after RYGB surgery in both the sNGT and sT2DM groups (n=15).
5.4.1 Differentially expressed genes after RYGB surgery that were confirmed by TLDA RT-qPCR

TLDA RT-qPCR gene expression analysis was restricted to the sNGT or sT2DM groups so as to control for the affect of weight loss versus the gene changes induced by improved insulin sensitivity or remission of diabetes. Genes that were significantly altered in only the sT2DM group, and tended to have a greater fold change in the sT2DM than the sNGT group are highlighted in Table 5-4. In this way we can highlight the genes most likely to be altered by the change in diabetic status as opposed to weight loss and other factors present in both groups.

Of the two confirmed genes that belonged to the glucose metabolism category, ENO3 had similar changes in gene expression in both the sNGT and sT2DM groups. Confirmed genes that were involved in lipid metabolism, which included LDLR, PLIN2, FADS and FASN, all had similar differential expression after RYGB surgery in both groups. The majority of the confirmed genes involved in regulating transcription (ATF5, CSRPNP1, RCAN1) and genes involved in cell growth and differentiation (DUSP1, TP53INP1, FNDC) had similar fold changes after RYGB surgery in both groups. Confirmed genes that are involved in cellular transport and cell structure (P4HA1, CRYAA, RPL10A and TMEM45B) also had similar changes in gene expression in both the sNGT and sT2DM group.

In some cases, however, individuals with type 2 diabetes tended to have a greater magnitude in differential gene expression. Many of the confirmed genes involved in inflammation and blood coagulation (CRP, SERPINE1, and A2M,) had a greater magnitude in fold change in individuals with type 2 diabetes after RYGB surgery. Likewise, fold changes in UPP2 expression was greater in individuals who had a remission of type 2 diabetes. Although most of the genes involved in transport had similar differential expression in both groups, expression of SLC2A3 and HBA1 was only significantly decreased in individuals who had a remission of type 2 diabetes.

Confirmed genes that are involved in xenobiotic metabolism (MT1H and MT1F) tended to have a greater decrease in gene expression in the sNGT group. The exception to this was CYP1A2, which was only significantly increased in the sT2DM group after RYGB surgery.
Table 5-4. Confirmed genes that were differentially expressed after RYGB surgery. Highlighted genes are likely affected by changes in diabetic or insulin-resistant status.

<table>
<thead>
<tr>
<th>Gene*</th>
<th>sNGT (Post/Pre)</th>
<th>sT2DM (Post/Pre)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fold Change</td>
<td>95% CI</td>
</tr>
<tr>
<td>INHBE</td>
<td>-2.5</td>
<td>-15.4 to 2.4</td>
</tr>
<tr>
<td>ENO3</td>
<td>-2.3</td>
<td>-8.1 to 1.5</td>
</tr>
<tr>
<td>LDLR</td>
<td>-2.6</td>
<td>-5.6 to -1.0</td>
</tr>
<tr>
<td>PLIN2</td>
<td>-1.8</td>
<td>-3.9 to 1.6</td>
</tr>
<tr>
<td>FADS</td>
<td>-1.5</td>
<td>-6.70 to 2.20</td>
</tr>
<tr>
<td>FASN</td>
<td>-1.7</td>
<td>-8.3 to 3.0</td>
</tr>
<tr>
<td>UPP2</td>
<td>-2.5</td>
<td>-12.3 to 1.8</td>
</tr>
<tr>
<td>CRP</td>
<td>-3.2</td>
<td>-23.3 to 1.9</td>
</tr>
<tr>
<td>IL1RN</td>
<td>-1.7</td>
<td>-6.8 to 2.2</td>
</tr>
<tr>
<td>IL1RAP</td>
<td>1.3</td>
<td>-2.4 to 3.6</td>
</tr>
<tr>
<td>SERPINE1</td>
<td>-6.9</td>
<td>-14.9 to 16.5</td>
</tr>
<tr>
<td>A2M</td>
<td>1.6</td>
<td>-2.6 to 6.5</td>
</tr>
<tr>
<td>ATF3</td>
<td>-4.6</td>
<td>-76.9 to 3.8</td>
</tr>
<tr>
<td>ATF5</td>
<td>-1.7</td>
<td>-5.4 to 1.6</td>
</tr>
<tr>
<td>BHLHE40</td>
<td>-1.5</td>
<td>-5.6 to 2.6</td>
</tr>
<tr>
<td>CSRN1</td>
<td>-2.3</td>
<td>-10.1 to 4.1</td>
</tr>
<tr>
<td>CDKN1A</td>
<td>-2.0</td>
<td>-6.0 to 1.3</td>
</tr>
<tr>
<td>RCAN1</td>
<td>-2.6</td>
<td>-12.5 to 2.2</td>
</tr>
<tr>
<td>DUSP1</td>
<td>-2.3</td>
<td>-8.8 to 1.7</td>
</tr>
<tr>
<td>FNDC5</td>
<td>-2.7</td>
<td>-13.2 to 1.3</td>
</tr>
<tr>
<td>TP53INP1</td>
<td>-2.3</td>
<td>-8.1 to 1.7</td>
</tr>
<tr>
<td>IGFB2</td>
<td>3.3</td>
<td>-2.5 to 24.1</td>
</tr>
<tr>
<td>MT1F</td>
<td>-3.1</td>
<td>-13.9 to 1.8</td>
</tr>
<tr>
<td>MT1H</td>
<td>-5.7</td>
<td>-52.6 to 1.8</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>1.1</td>
<td>-4.5 to 4.2</td>
</tr>
<tr>
<td>SLC2A3</td>
<td>-1.7</td>
<td>-7.5 to 1.4</td>
</tr>
<tr>
<td>SHBG</td>
<td>1.8</td>
<td>-2.1 to 8.2</td>
</tr>
<tr>
<td>OSTalpha</td>
<td>1.7</td>
<td>-1.5 to 4.0</td>
</tr>
<tr>
<td>P4HA1</td>
<td>3.0</td>
<td>-3.1 to 12.9</td>
</tr>
<tr>
<td>CRYAA</td>
<td>-1.5</td>
<td>-7.9 to 3.6</td>
</tr>
<tr>
<td>HBA1</td>
<td>-1.6</td>
<td>-13.2 to 3.5</td>
</tr>
<tr>
<td>RPL10A</td>
<td>1.3</td>
<td>-1.3 to 2.5</td>
</tr>
<tr>
<td>SERPINA1</td>
<td>1.4</td>
<td>-2.2 to 2.9</td>
</tr>
<tr>
<td>TMEM154</td>
<td>-2.1</td>
<td>-10.8 to 1.9</td>
</tr>
<tr>
<td>TMEM45B</td>
<td>-2.1</td>
<td>-13.0 to 4.2</td>
</tr>
</tbody>
</table>

* Gene fold change, confidence interval and p-values were calculated using REST 2009.
Several genes that were differentially regulated after RYGB surgery belong to biological processes involved in type 2 diabetes or were affected by presence of diabetes. Figure 5.4-2 presents the mean gene expression ($2^{-\Delta Ct}$) of these genes relative to 18s before and after RYGB surgery. INHBE codes for a secreted protein involved in regulation of insulin secretion. Mean INHBE expression tended to be higher in the sT2DM group before RYGB surgery, although this did not reach statistical significance. INHBE expression had a statistically significant decrease in both groups, while individuals with type 2 diabetes tended to have a greater decrease in INHBE expression (Figure 5.4-2). After RYGB surgery, INHBE expression was the same in both the sNGT and sT2DM group. IGFB2 expression tended to be lower in the sT2DM group before RYGB surgery (p=0.09, students t-test). Although both the sNGT and sT2DM group had a significant increase in IGFB2 expression, the magnitude of the increase was greater in individuals who had type 2 diabetes (Figure 5.4-2). Expression of IL1RAP was also lower in the sT2DM group before RYGB surgery (p=0.01, students t-test) and had a statistically significant increase after RYGB surgery in the sT2DM group only (Figure 5.4-2).

ATF3, BHLHE40 and CDKN1A all appeared to be affected by presence of diabetes. ATF3 is involved in regulation of gluconeogenesis and it tended to be expressed at a higher level in the sT2DM group, although this was not statistically significant. However, individuals with type 2 diabetes had a -11.36 fold decreased in ATF3 expression, which was higher than the -5.6 fold decrease seen in the sNGT group. Expression of BHLHE40 also tended to be higher in the sT2DM group at the time of RYGB surgery (p=0.07, students t-test) and the change in expression after RYGB surgery was only statistically significant in the sT2DM group. The magnitude of the decrease in the sT2DM group (-3.1 fold) was greater than that seen in the sNGT group (-1.5 fold), which was statistically significant (p=0.04, 2 way ANOVA). Although CDKN1A expression was decreased to a similar extent in both groups, the sT2DM group had a statistically significant (p=0.006) higher expression even after RYGB surgery (Figure 5.4-2).
Figure 5.4.2 Mean ± SE INHBE, IGFB2, ATF3, BHLHE40, CDKN1A, and IL1RAP expression presented as $2^{-\Delta\Delta Ct}$ normalised to 18S in the sNGT (●, n=8) and the sT2DM (○, n=8) group before and after RYGB surgery. *Gene expression change was statistically significant (p<0.05, paired t-test)).
5.4.2 Differentially expressed genes between individuals with normal glucose tolerance and type 2 diabetes that were confirmed by TLDA RT-qPCR

Genes that were found to be differentially expressed between the sNGT and sT2DM with microarray analysis were also confirmed with TLDA RT-qPCR. Figure 5.4-3 presents gene expression relative to 18S. Regardless of whether they had RYGB surgery, individuals with type 2 diabetes had significantly lower hepatic mRNA expression of \textit{CYP2C19} (-2.9 fold, 95% CI -11.5 to 2.0), \textit{DBH} (-2.7 fold, 95% CI -25 to 2.3) and \textit{IGFALS} (-1.6 fold, 95% CI -6.0 to 1.9) in comparison to individuals with normal glucose tolerance (Figure 5.4-3). Conversely, hepatic mRNA expression of \textit{IGJ} (2.6 fold, 95% CI -2.2 to 20.3), \textit{LGALS4} (2.5 fold, 95% CI -1.68 to 10.0), \textit{PYROXD2} (3.4 fold, 95% CI -1.3 to 11.2) and \textit{SAA1} (3.0 fold, 95% CI -2.3 to 15.9) was significantly elevated in individuals with type 2 diabetes (Figure 5.4-3).

![Graphs showing gene expression](image)

Figure 5.4-3. Mean ± SE \textit{CYP2C19}, \textit{DBH}, \textit{IGFALS}, \textit{IGJ}, \textit{LGALS4}, \textit{PYROXD2}, and \textit{SAA1} expression ($2^{-\Delta C_{t}}$) in the sNGT ($\bullet$, n=8) and the sT2DM ($\circ$, n=8) before and after RYGB surgery. *The difference in gene expression was statistically significant (p<0.05, t-test).
5.4.3 LGALS4 protein abundance is elevated in individuals with type 2 diabetes

Elevated gene mRNA expression does not always translate directly to elevated protein abundance. LGALS4 protein abundance was assayed by western blotting to further confirm the quality of the microarray data. LGALS4 protein abundance was assayed in the study groups used previously in this thesis which consisted of individuals with normal glucose tolerance (NGT) or type 2 diabetes (T2DM). Individuals with type 2 diabetes tended to have higher abundance of LGALS4 protein in comparison to individuals with normal glucose tolerance (Figure 5.4-4). LGALS4 may be related to increased concentrations of carbohydrates like glucose. Out of the 5 clinical parameters recorded (HOMA-IR, BMI, HbA1c, fasting plasma insulin and glucose), LGALS4 protein abundance only positively correlated with fasting plasma glucose.

Figure 5.4-4. (A) Mean + SE liver LGALS4 protein abundance (relative to actin) of NGT (n=17) and T2DM (n=26) groups. See Appendix for complete western blot. (B) Plot of liver LGALS4 protein relative abundance (log) vs fasting plasma glucose concentration (log).
5.5 Discussion

The progression from insulin resistance to frank hyperglycaemia and type 2 diabetes is a long and complex process. Although animal models have advanced our understanding of this progression, lack of human studies in key tissue such as the liver has limited our understanding of the holistic view of type 2 diabetes pathogenesis. Furthermore, limited access to tissue such as liver may have hindered the recognition of novel genes or molecular pathways involved in this disease. RYGB surgery improves insulin sensitivity and thus provides a model that can help identify novel genes related to insulin resistance or type 2 diabetes.

In this study, we used microarray analysis to assay global liver gene expression at RYGB surgery and ~16 months later. All individuals had a significant decrease in fasting plasma insulin levels and an improvement in insulin sensitivity. Individuals who had type 2 diabetes at RYGB surgery were in remission by the second operation. Grouping individuals according to diabetic status allowed us to identify candidate genes affected by presence or absence of hyperglycaemia and hyperinsulinemia. A complete review of differential gene expression after RYGB surgery and how it relates to the phenotypical changes seen in type 2 diabetes is available in Chapter 6.

The following discussion is focused on genes that were confirmed with TLDA RT-qPCR and were differentially regulated after remission of type 2 diabetes. In all cases TLDA RT-qPCR showed a greater change in gene expression, most likely related to the saturation of the beads on the microarray chip, and the higher dynamic range inherent in RT-qPCR.

A key observation in this study was differential expression of genes that code for secreted proteins involved in mediating insulin secretion or glucose homeostasis. Expression of a recently identified activin, INHBE, tended to be higher in individuals with type 2 diabetes and decreased after RYGB surgery (Figure 5.4-2). The liver secretes INHBE in response to insulin stimulus, which then goes on to act on the pancreas to inhibit insulin secretion. These data suggest that in a natural state, INHBE acts as a break for excessive insulin secretion. However because of the hyperinsulinaemia present in individuals with insulin resistance, pathological overexpression of INHBE may result in negative effects on the pancreatic cell.
Indeed, overexpression of INHBE in mice undermined growth of pancreatic exocrine cells.573 Raised expression of INHBE in individuals with marked insulin resistance may contribute to the gradual loss of beta cell function that eventually precipitates overt diabetes. DeFronzo and Abdul-Ghani (2011) have suggested that development of pharmacological intervention that preserves 20-30% of pancreatic β-cell function would prevent progression from impaired glucose tolerance to type 2 diabetes.229 INHBE is a promising target for such treatments.

IGFBP2 is another gene secreted from the liver that had differential expression after RYGB surgery. IGFBP2 has a role in mediating metabolic homeostasis574 and investigators have documented increased expression after significant weight loss following RYGB surgery.545 In this study, IGFBP2 expression tended to be lower in individuals with T2DM at RYGB surgery in comparison to individuals who had normal glucose tolerance and increased 7.5 fold after remission of diabetes. Our results agree with that of Li et al. (2012) who showed circulating IGFBP2 increased within 24 hours and remained raised up to a year after bariatric surgery.575 Several animal models have shown IGFBP2 to have anti-diabetic effects.576, 577 A study by Hedbacker et al. (2010) found liver-specific IGFBP2 overexpression reversed diabetes in insulin resistant or obese mice.576 The same study also found that leptin increased IGFB2 levels, suggesting that IGFBP2 signalling is responsible for leptins effect on metabolic homeostasis.576 These findings have great implications given that raised CRP levels induce leptin resistance by inhibiting leptin signalling.578 Notably, CRP had decreased expression after RYGB surgery, particular in those individual who had remission of T2DM (Table 5-4), while in the same individuals IGFBP2 expression increased. This presents a potential mechanism whereby inhibition of leptin signalling by CRP decreases IGFBP2 levels resulting in worsened glycaemic control.

Multiple investigators have used genome wide association studies to identify up to 18 genes that are consistently associated with type 2 diabetes.584, 585 Several of these genes mediate pancreatic β-cell function, which is in agreement with the differential INHBE expression observed by us and with the hypothesis that β-cell dysfunction is critical for progression to type 2 diabetes.579, 580
Of all the genes identified by us, only \textit{IGFBP2} has been associated with type 2 diabetes, and several genome-wide association studies having identified polymorphisms near the \textit{IGFBP2} gene as a risk factor for type 2 diabetes.\textsuperscript{581, 582}

Because RYGB surgery causes dramatic changes in intestinal physiology, identifying genes directly involved in regulation of metabolism can be problematic. Indeed, expression of \textit{SDS} decreased after RYGB surgery most likely due to dietary cobalamin (vitamin B12) deficiency,\textsuperscript{583} which is a well known complication of bariatric surgery.\textsuperscript{584-586} Confirmed genes had similar expression regardless of diabetic status, suggesting that changes in gut physiology, dietary habits and weight loss are responsible for most of the differential gene expression. Both our study and that of Elam et al. (2009) showed changes in expression of liver genes involved in lipid metabolism, inflammation, cell structure, xenobiotic metabolism and cell growth after RYGB surgery. Similarly, a study by Tamboli et al. (2011) exploring differential gene expression in skeletal muscle after RYGB surgery with or without omentectomy showed a decrease in inflammatory gene expression.\textsuperscript{544} Unlike previously published studies however, our cohort clearly distinguished between individuals with normal glucose tolerance or type 2 diabetes. Availability of liver tissue from the same individual before and after significant increases in insulin sensitivity allowed us to identify many differentially expressed genes that belonged to pathological processes associated with T2DM.

Although both groups had a decrease in \textit{CDKN1A} expression after RYGB surgery, its expression was higher in individuals who had type 2 diabetes. A study by Scott-Drecshel et al. (2013) associated hyperglycaemia with increased \textit{CDKN1A} expression, which caused a slowed cell cycle progression leading to reduced cellular proliferation.\textsuperscript{587} Indeed, genes such as \textit{DUSP1}, \textit{TP53INP1}, \textit{GADD45B}, and \textit{DDIT4} are all involved in negative regulation of cell growth and were decreased after RYGB surgery. Individuals with type 2 diabetes tended to have a greater fold change in genes that regulate inflammation or coagulation (\textit{CRP}, \textit{SERPINE1}, and \textit{A2M}). \textit{IL1RN} and \textit{IL1RAP}, both of which are involved in the IL-1 signalling pathway, were differentially expressed after RYGB surgery. IL-1 is a proinflammatory cytokine that plays a central role in chronic inflammatory diseases\textsuperscript{588} and has recently been linked to fat-liver cross talk in obesity.\textsuperscript{350} \textit{IL1RN} is thought to inhibit proinflammatory IL-1
signalling by binding to the IL-1 receptor and preventing its association with the IL1RAP coreceptor.\textsuperscript{589-591} IL1RN has been found to be increased in diet-induced obesity,\textsuperscript{592, 593} and is likely increased to counter the proinflammatory state observed in obesity and type 2 diabetes. The decrease in \textit{IL1RN} expression after RYGB surgery is consistent with the marked weight loss and improvement in inflammatory state that has been documented by others.\textsuperscript{594, 595} IL1RAP is an essential component required for IL-1 signalling through the IL-1 receptor\textsuperscript{596, 597} and has been implicated in regulation of insulin sensitivity, pancreatic β-cell mass and leptin resistance.\textsuperscript{592, 598} \textit{IL1RAP} had a lower level of expression in individuals with type 2 diabetes and was increased after RYGB surgery individuals who experienced remission of type 2 diabetes.

Several microarray studies have shown that insulin regulates a vast array of gene expression.\textsuperscript{548, 599-602} There are many similarities between liver biological processes that are affected by RYGB and those that change in skeletal muscle tissue after insulin infusion.\textsuperscript{548, 600} In both instances, a large portion of the differential gene expression was observed in genes which code for proteins that regulate transcription factor binding or activity. Coletta \textit{et al.} (2008)\textsuperscript{548} identified 12 genes that were differentially regulated in parallel with our study, while four of those genes (\textit{ATF3}, \textit{FOSB}, \textit{BHLHB40}, and \textit{TRIB1}) were involved in regulation of transcription. The similarity between the two studies is not surprising as all individuals in this study had a decrease in circulating insulin concentrations, which was more pronounced in those who experienced remission of type 2 diabetes (Table 2-3).

However, differential regulation of \textit{ATF3} and \textit{BHLHE40} in the liver is more relevant to glucose homeostasis. In this study, gene expression of \textit{ATF3} and \textit{BHLHE40} tended to be higher in individuals with type 2 diabetes. Interestingly, increased \textit{ATF3} expression in the liver of transgenic mice led to impaired glucose homeostasis because of its ability to inhibit gluconeogenesis.\textsuperscript{603} \textit{ATF3} expression was decreased by -11.4 fold in individuals who experienced remission of type 2 diabetes whereas it was decreased -4.6 fold in those individuals that only had an improvement in insulin sensitivity. Decreased \textit{ATF3} expression is likely related to the changes in insulin sensitivity, although the exact mechanism that involves \textit{ATF3} regulation of gluconeogenesis remains unknown.
BHLHE40 (alternatively known as BHLHB2) is a pleiotropic transcription factor that has roles in cellular process that range from metabolism to regulation of circadian rhythm.\textsuperscript{604} Like, \textit{ATF3} Expression of \textit{BHLHE40} was decreased to a greater extent in individuals who had remission of type 2 diabetes. Although \textit{BHLHE40} expression is increased by insulin,\textsuperscript{548, 599, 600, 605} glucose is also able to induce \textit{BHLHE40} expression through ChREBP activation.\textsuperscript{606} Consequently, the resolved hyperinsulinemia and hyperglycaemia observed in individuals who have a remission of diabetes may be responsible for the decreased \textit{BHLHE40} expression. Overexpression of \textit{BHLHE40} in individuals with type 2 diabetes has several implications. First, BHLHE40 was found to inhibit glucose and ChREBP-mediated activation of lipogenic genes, suggesting it may play an important role in preventing excessive lipogenesis.\textsuperscript{606} Second, \textit{BHLHE40} may have an effect on regulating hepatic glucose metabolism through its ability to suppress CLOCK/BMAL1 enhanced promoter activity.\textsuperscript{607} CLOCK and BMAL-1 constitute the core of the molecular clock and have been found to regulate glucose metabolism at the liver.\textsuperscript{608-610} The relationship between metabolism and the circadian rhythm is further strengthened by recent data that shows insulin action has a circadian rhythm, which when disrupted caused predisposition to obesity and insulin resistance.\textsuperscript{611} Differential \textit{BHLHE40} expression after remission of diabetes is consistent with the increasing relevance of circadian rhythm disruption in type 2 diabetes.\textsuperscript{612, 613}

The cohort used in this study also offered the opportunity to identify differential liver gene expression between individuals with or without type 2 diabetes. \textit{DBH} was expressed at a lower level in individuals with type 2 diabetes and has previously been associated with diabetes.\textsuperscript{614} Expression of \textit{SAA1} was increased in individuals with type 2 diabetes and has been proposed as a potential biomarker for insulin resistance.\textsuperscript{615} LGALS4 and PYROXD2 had higher expression in individuals with T2DM regardless of whether they underwent RYGB surgery and are for the first time associated with T2DM. In addition, LGASL4 protein was confirmed to be elevated in a different set of individuals with type 2 diabetes and had a positive correlation with glucose (Figure 5.4-4). LGALS4 (also known as galectin-4) is a part of the galectin subfamily, which are carbohydrate binding proteins with diverse functions that range from cell adhesion to intercellular signalling.\textsuperscript{616} LGALS4 mediates intestinal inflammation\textsuperscript{617} and has a role in innate immunity because it binds to carbohydrate
moieties on the surface of bacteria resulting in rapid loss of viability.\textsuperscript{618, 619} Although the functional characteristics of PYROXD2 are relatively unknown, its ability to metabolise trimethylamine suggests a role in the interactions between the gut microbiome and host.\textsuperscript{620, 621} Interestingly, the interactions between gut flora and host metabolism have become topical for metabolic disease, while changes in gut flora after RYGB surgery suggest the microbiome has an important role in T2DM pathogenesis.\textsuperscript{622, 623}

To conclude, although RYGB surgery induces multiple changes in gene expression, by restricting analysis of these changes to those individuals with or without type 2 diabetes we have identified genes that may have a key role in the progression of insulin resistance to type 2 diabetes. Of all the genes changes observed, \textit{INHBE} is of particular interest as a potential therapeutic target that might be critical to preventing or delaying progression from insulin resistance to type 2 diabetes. Decreased expression of \textit{INHBE} in the liver after RYGB may again highlight the importance of liver insulin resistance in type 2 diabetes and provides evidence for a hitherto unrecognised mechanism by which the liver regulates insulin secretion.

Decreased expression of \textit{BHLHE40} only in those individuals who had remission of type 2 diabetes provides a link between disruption of the circadian rhythm and dysregulated metabolic homeostasis. This finding is particularly relevant given the recent evidence showing insulin action to have a circadian rhythm and the increasing recognition of the association between circadian rhythm disruption and insulin resistance. The expression of several genes was markedly different between individuals with normal glucose tolerance or type 2 diabetes. Although many of these, including \textit{LGALS4} and \textit{PYROXD2}, have no known relationship to type 2 diabetes, the increasing awareness of the gut microbiome and its relevance to metabolic homeostasis may be of real importance.
CHAPTER SIX: Global changes in hepatic gene expression after RYGB surgery reflect the pathology of type 2 diabetes
Obesity-associated type 2 diabetes is a polygenic disease caused by a combination of environmental and genetic factors. The central notion governing development of type 2 diabetes is the progressive insulin resistance that causes impaired glucose tolerance eventually leading to pancreatic β-cell failure and fasting hyperglycemia. In the last 50 years there has been a growing knowledge of the molecular processes that drive the progression to type 2 diabetes. Chief among them are lipotoxicity and inflammation, both of which are thought to contribute equally to the development of insulin resistance.

RYGB surgery induces drastic changes in diet and involves significant manipulation of the gastrointestinal system, so it is not surprising that there are changes in gene expression regulating a wide range of biological processes. Genes such as LDLR, FASN, PLIN2, SQLE, FADS1 and ACSL4 regulate lipid biosynthesis or transport and were downregulated after RYGB surgery. This mirrors the dramatic weight loss seen after surgery and is likely associated with the decreased caloric intake or change in diet. Genes involved in cell adhesion such as CNTNAP2 and RND3 were downregulated, whereas genes involved in extracellular matrix remodelling such as PTHA1 were upregulated after RYGB surgery. Infiltration of the liver by FFA, prior to RYGB, leading to non-alcoholic fatty liver disease (NAFLD) may cause differential regulation of genes involved in cell adhesion and ECM remodelling. Several studies investigating gene expression in NAFLD have linked genes involved in liver cell adhesion and lipid metabolism with increased liver fat content.

Although oxidative stress is recognized as another contributing factor in diabetes, only in the last decade has endoplasmic reticulum stress and more specifically, the unfolded protein response (UPR) been acknowledged as a key factor in development of insulin resistance. Similarly, the disruption of the circadian rhythm and the gut microbiome are also increasingly becoming recognized as key factors that contribute to development of type 2 diabetes. This chapter provides a brief literature review of the key gene changes observed in liver tissue after RYGB surgery or after remission of type 2 diabetes and how they relate to these pathological processes.
6.1 Glucose metabolism

Dysregulation of gluconeogenesis is thought to contribute to the fasting hyperglycaemia seen in type 2 diabetes. SIK1 and ATF3 negatively regulate gluconeogenesis and have diminished expression after RYGB surgery. SIK1 is a part of the SIK family proteins that have a wide variety of biological functions including an increasingly important role in metabolic regulation. SIK1 has been shown to inhibit gluconeogenesis by regulating CREB-mediated transcription of gluconeogenic genes and lipogenesis by regulating SREBP-1c-mediated transcription of lipogenic genes. ATF3 also inhibits gluconeogenesis by repressing transcription of PCK1. The decreased expression of SIK1 and ATF3 is likely associated with the normalisation of overactive lipogenesis and gluconeogenesis seen after RYGB surgery.

PCK1 positively regulates gluconeogenesis because it codes for an enzyme (PEPCK) that catalyzes one of the first steps in the gluconeogenic reaction. The inhibitory action of insulin on PCK1 transcription has been well established, while silencing PCK1 expression in the liver has been shown to improve glucose homeostasis, insulin sensitivity and dyslipidemia in diabetic mice. PCK1 expression was only increased in those individuals who experienced remission of diabetes (Table 5-2). This is likely linked to the decreased expression of ATF3, which was more pronounced in individuals who had remission of type 2 diabetes. Even so, the increased expression of PCK1 after normalisation of hyperglycaemia initially appears to be paradoxical.

It is critical to note, however, that both the pre and post RYGB liver biopsies were taken under fasting conditions and there is evidence to suggest that insulin regulation of PCK1 transcription may differ in the fed and fasting state. Although the ability of insulin to regulate PCK1 mRNA expression was impaired in ad libitum insulin-resistant Zucker fatty rats, the impairment was partially restored after an overnight fast. Furthermore, individuals in this study who had remission of diabetes also displayed a marked decrease in fasting insulin concentration which likely had a role in inhibiting PCK1 transcription. Taken together these data suggest that regulation of gluconeogenesis is a complex process that is regulated by multiple checks and counter
balances. Elucidating how SIK1, ATF3 and PCK1 act in concert to regulate gluconeogenesis will aid in the understanding the pathogenesis of type 2 diabetes.

6.2 Regulation of metabolic homeostasis via leptin signalling

Leptin signalling through the leptin receptor (LEPR) is involved in satiety and metabolic homeostasis. Expression of LEPR is decreased after RYGB surgery. In healthy individuals leptin prevents obesity via LEPR by stimulating glucose uptake and fatty acid oxidation. Several mutations of the LEPR gene have been associated with type 2 diabetes and the metabolic syndrome. Interestingly, the liver was recently suggested to have a role in modulating leptin action via the production of soluble leptin receptors. LEPR expression was shown to increase after treating mice with leptin which consequently increased soluble leptin receptors levels. Obesity is associated with increased levels of circulating leptin and bariatric surgery has been shown to significantly decrease leptin levels. It can be inferred that the decreased LEPR expression observed in this study is likely linked to the decreased level of leptin seen after bariatric surgery. How this affects leptin signalling is yet to be determined. Nonetheless, leptin signalling is known to have a significant role to play in type 2 diabetes and the associated metabolic syndrome. Specifically, increased levels of circulating leptin in the face of obesity have inspired the concept of “leptin resistance”. Akin to insulin resistance, leptin resistance may have a role to play in insulin sensitivity.

Data presented in Chapter 5 provides evidence for a hitherto unconfirmed relationship between leptin, CRP and IGFB2. Elevated CRP levels have been shown to induce leptin resistance by inhibiting leptin signalling. Notably, expression of CRP was decreased after RYGB surgery, particular in those individual who had remission of type 2 diabetes (Table 5-4, Chapter 5). Although its expression is increased in liver and adipose tissue in severely obese individuals, its role in type 2 diabetes is controversial. Some studies have shown CRP to be associated with increased risk of developing type 2 diabetes, while others have not.
Even though the mechanism behind the involvement of CRP in type 2 diabetes is yet to be elucidated, its ability to inhibit leptin signalling\textsuperscript{578} is an interesting finding in light of a recently identified relationship between leptin and IGFB2 levels.

Leptin has been shown to increase levels of IGFB2,\textsuperscript{576} which has a well documented role in glucose homeostasis.\textsuperscript{574} In this study, \textit{IGFBP2} expression was increased after RYGB surgery, which again was more pronounced in those individuals who had remission of type 2 diabetes. A likely scenario in individuals with type 2 diabetes may be that elevated levels of CRP inhibit leptin signalling, thus decreasing IGFB2 levels which would have detrimental affect on glucose homeostasis. Accordingly, data presented in Chapter 5 show elevated expression of \textit{CRP} and depressed expression of \textit{IGFB2} during diabetes, which is normalised after RYGB surgery and remission of diabetes. The observation that there is a relationship between leptin, CRP, and IGFB2 is in accord with the increasingly evident relationship between inflammation and metabolic homeostasis.\textsuperscript{303, 655}

\section*{6.3 Lipotoxicity and non-alcoholic fatty liver disease}

Dysfunctional lipid homeostasis in the context of obesity has been recognized as a key pathology in type 2 diabetes. Accumulation of lipids in insulin target tissues, otherwise known as lipotoxicity, disrupts insulin signalling and is one of the contributing factors that lead to insulin resistance. In the liver, lipotoxicity manifests itself as non-alcoholic fatty liver disease (NAFLD),\textsuperscript{656} which when reversed has been shown to improve insulin sensitivity and normalize hyperglycemia.\textsuperscript{256, 257} Similarly, NAFLD has been shown to improve after bariatric surgery and weight loss.\textsuperscript{657, 658}

In this study several genes that are associated with lipid homoeostasis and accumulation were differentially regulated after RYGB surgery. Sterol regulatory binding proteins (SREBPs) are transcription factors central to mediating lipid catabolism and lipid biogenesis in the liver.\textsuperscript{200, 320} They regulate up to 30 genes directly involved in uptake and synthesis of fatty acids, triglycerides, cholesterol and phospholipids.\textsuperscript{200}
LDLR and FASN are involved in the SREBP pathway and were both decreased after RYGB surgery. The LDLR gene codes for a cell surface glycoprotein that is involved in mediating blood cholesterol levels by removing LDL particles from the circulation. Insulin has been shown to mediate the levels of LDLR expression via its influence on SREBP-1 which in turn mediates activation of the LDLR promoter. Furthermore, LDLR has been found to associate with the insulin receptor which diminishes its ability to bind LDL particles. Insulin stimulation causes disassociation of the LDLR and IR receptor thereby increasing the capacity of LDLR to bind LDL particles. FASN catalyzes the last step in fatty acid biosynthesis and is regulated by SREBP. Increased FASN expression in adipose tissue has been linked with type 2 diabetes while increased FASN expression has also been shown in NAFLD. Consequently the decreased expression of LDLR and FASN is likely associated with the decreased levels of insulin seen after RYGB surgery and the consequent improvement in lipid homeostasis.

PLIN2, a marker of lipid accumulation, had decreased expression after RYGB surgery and remission of diabetes. PLIN2 coats lipid droplets and is thought to be involved in their intracellular mobilization. Lipid droplets are intracellular and cytoplasmic structures that store triglycerides in times of energy abundance. They are implicated in metabolic disease and their role in diabetes and NAFLD is well documented. Increased PLIN2 expression is associated with increased lipid droplets and fatty liver in both humans and animal models. PLIN2 overexpression increased lipid accumulation without induction of other lipogenic genes, suggesting PLIN2 alone is capable of causing lipid accumulation in cells. Conversely, PLIN2 null mice were resistant to diet-induce fatty liver and obesity, while knocking down PLIN2 in obese mice reversed hepatic steatosis, hypertriglyceridemia and insulin resistance. This evidence, along with our data, suggests PLIN2 may be involved in diabetes-associated lipotoxicity.

The exact mechanisms behind the negative affect of NAFLD on insulin regulation of glucose homeostasis are still unknown. Nonetheless, KLF6 has been identified as a candidate molecule that links NAFLD to dysregulated glucose homeostasis. In this study KLF6 expression was decreased after RYGB surgery (Table 5-2, Chapter 5).
KLF6 is a pleiotropic transcription factor that was shown to be involved in the regulation of cell proliferation, differentiation and tumorigenesis.\textsuperscript{683} It was found to be rapidly induced in hepatic stellate cells after injury\textsuperscript{684} and has since been associated with liver fibrosis and NAFLD.\textsuperscript{685, 686} KLF6 was recently implicated in regulation of hepatic glucose metabolism and insulin sensitivity via its interaction with glucokinase.\textsuperscript{687} Furthermore, elevated KLF6 expression was associated with elevated activation of PPAR\textsubscript{α} signalling.\textsuperscript{688} Because PPAR\textsubscript{α} deficiency was shown to protect mice against insulin resistance induced by a high-fat diet, it can be inferred that elevated expression of \textit{KLF6} has a detrimental affect on glucose and lipid homeostasis.\textsuperscript{689}

\section*{6.4 Inflammation and blood coagulation}

Obesity-induced inflammation can occur via several mechanisms. Recruitment of adipose tissue macrophages (ATMs) by chemokine signalling is thought to play a key role.\textsuperscript{280-283} FFAs and other metabolites like glucose are also thought to have a role in activating the inflammasome,\textsuperscript{303} which leads to induction of NF-\textkappa\textbeta,\textsubscript{333} TNF\textalpha and IL-1\beta among others.\textsuperscript{333, 334} Furthermore, adipose derived IL-1\beta was shown to act on the liver by supporting ectopic lipid accumulation in hepatocytes.\textsuperscript{350}

In this study, multiple genes that have been shown to be involved in inflammation were differentially regulated after RYGB surgery. \textit{IL1RN} and \textit{IL1RAP}, both of which modulate the strength of IL-1 signalling, were differentially regulated after RYGB surgery. This is likely associated with a possible decrease of activity in the IL-1 signalling pathway. Expression of \textit{IL-32} was also decreased after RYGB surgery. IL-32 is a recently identified proinflammatory cytokine that increases expression of TNF\textalpha and IL-1\beta and has been found to have an auto-inflammatory relationship via its synergy with TNF\textalpha.\textsuperscript{690} The decrease in \textit{IL32} expression after RYGB surgery is consistent with the improvements observed in metabolic homeostasis.

Dysregulated blood coagulation is another pathological condition seen in obesity-related type 2 diabetes. Cardiovascular risk is increased in individuals with type 2 diabetes\textsuperscript{691} while changes in the homeostatic mechanism have long been associated with this disease.\textsuperscript{692, 693}
In this study, expression of SERPINE-1 (alternatively known as PAI-1) was decreased after RYGB surgery and remission of type 2 diabetes. SERPINE-1 is a circulatory protein that is involved in regulation of the fibrinolytic system in blood by inhibiting both urokinase-type and tissue-type plasminogen activators. Regulation of SERPINE-1 transcription is under the control of a vast array of stimuli, including proinflammatory cytokines, TNFα, lipids, growth factors and insulin. Dysregulation of the fibrinolytic system, often seen in type 2 diabetes and its complications, is associated with increased levels of SERPINE-1 expression.

Hyperinsulinemia has long been suspected as a risk factor for the development of vascular disease and SERPINE-1 was singled out as a major player. The connection between hyperinsulinemia and insulin induction of SERPINE-1 expression may be a possible mechanism that links obesity, diabetes and cardiovascular disease.

The effects of inflammation are partially mediated by activation of transcription factors that increase expression of proinflammatory cytokines. Enhanced activation of liver NF-κB and activating protein-1 (AP-1) was observed in obese patients with NAFLD, and both were positively correlated with insulin resistance. AP-1 activity can be induced by a complex set of factors that includes growth factors such as insulin and pro-inflammatory factors such as IL-1. The AP-1 family consists of homodimers and heterodimers of Jun (v-Jun, c-Jun, JunB, JunD), Fos (v-Fos, c-Fos, FosB, Fra1, Fra2) or activating transcription factor (AFT2, ATF3, B-ATF, JDP1 and JDP2). In this study, several members of the family of transcription factors were differentially regulated after RYGB surgery. Gene expression of c-JUN, JUND, FOSB, and ATF3 were all decreased after RYGB surgery.

AP-1 and its constituents control a wide variety of biological processes including cell proliferation, differentiation, apoptosis. JUN is a key activator of hepatocyte proliferation and is involved in liver regeneration, while JUND and FOSB were shown to be involved in wound healing and liver fibrosis. Furthermore, JUN and JUND were shown to be critical in protecting cells from oxidative stress. As oxidative stress has been linked to the pathogenesis of insulin resistance and type 2 diabetes, it is highly likely that the changes seen in AP-1 expression may be related to improvement in the inflammatory state and oxidative stress.
Like AP-1, ATF5 expression, which is also induced by a variety of cellular stresses,\textsuperscript{712} was decreased after RYGB surgery. Although little is known of the functional consequences of ATF5 induction in the liver, it has been shown to be involved in cellular survival,\textsuperscript{713} proliferation and differentiation.\textsuperscript{712, 714, 715} In the liver ATF5 has been shown to play a key role in modulating expression of CYP2B6 under stress conditions such as amino acid limitation or chemically-induced stress.\textsuperscript{716} Increased expression of ATF5 induced an increase in expression of CY2B6, a member of the cytochrome P450 family that is involved in oxidative metabolism of endobiotic/xenobiotics.\textsuperscript{716}

Several members of the metallothionein family had decreased gene expression after RYGB surgery (Table 5-2). Metallothioneins (MT) have a high affinity for metal ions and have been implicated in regulation of zinc and copper metabolism in addition to having anti-oxidant properties which protect against oxidative damage.\textsuperscript{717} A study by Coletta \textit{et al.} (2008), in which healthy individuals were treated with supraphysiological levels of insulin for four hours, showed that gene expression of MT proteins in muscle was increased following insulin stimulation.\textsuperscript{548} This suggested that insulin alone can activate the anti-oxidant defence system and may be a compensatory mechanism to the oxidative stress caused by increased free radicals from autoxidation of sugars and unsaturated lipids.\textsuperscript{710} MT’s may also play an important role in metabolic homeostasis. Polymorphisms in MT genes, and in particular \textit{MT1A}, were associated with type 2 diabetes mellitus,\textsuperscript{718} while \textit{MT2A} gene expression was found to be increased in adipose tissue of individuals with type 2 diabetes in comparison to lean individuals.\textsuperscript{719}

Further investigations revealed that TNFα increased \textit{MT2A} mRNA levels and treating 3T3-L1 cells with MT2A protein significantly inhibited glucose uptake.\textsuperscript{719} There is good evidence to suggest that MT’s have a significant role to play in the pathogenesis of insulin resistance and type 2 diabetes, but whether this role is central to the development or remission of the disease is yet to be established.
Oxidative stress caused by over-nutrition induces anti-oxidant and inflammation pathways. Until relatively recently the mechanism linking lipotoxicity and inflammation to insulin resistance had eluded the scientific community. In the last decade, however, endoplasmic reticulum stress has emerged as a key process in obesity and diabetes.308, 720

6.5 Endoplasmic reticulum stress

The endoplasmic reticulum (ER) is responsible for protein, lipid and sterol synthesis. In addition, it mediates protein folding and post translational modifications, both of which are necessary in order for proteins to carry out their cellular functions.721 Stressful conditions, such as hyperinsulinemia or excess nutrient intake, cause overproduction of proteins, which overloads the ER and leads to accumulation of unfolded proteins. This in turn activates the unfolded protein response (UPR),309 which acts to alleviate the unfolded protein overload via several responses. First it prevents the accumulation of further unfolded proteins by decreasing protein synthesis.722 Second, it increases expression of chaperon proteins that accelerate protein folding.721, 723 Finally, if ER function is severely impaired, the organelle elicits apoptotic signals that eliminate damaged cells.724, 725

ER stress and the UPR have been recognised as key processes in type 2 diabetes that link obesity-related inflammation to insulin resistance.244, 308, 720 FFAs were shown to induce ER stress in cultured hepatocytes and pancreatic β cells,726 whereas multiple markers of ER stress were activated in adipose tissue of obese individuals.727 Furthermore, ER stress was shown to decrease in adipose and liver tissue of individuals who had substantial weight loss after bariatric surgery.326 Similarly, in this study several genes that are involved in processes regulated by the UPR were differentially expressed after RYGB surgery. Expression of EEF1G, which mediates elongation during protein translation, was increased after RYGB surgery. Depressed EEF1G expression in the presence of obesity and type 2 diabetes is consistent with the involvement of ER stress and its ability to decrease protein synthesis.722 Expression of BRSK2 and DDIT4, both of which were shown to be regulated by ER stress,728, 729 was decreased after RYGB surgery.
DDIT4 is a stress response gene activated by hypoxia and other stimuli.\textsuperscript{730-732} Insulin has been shown to induce \textit{DDIT4} expression in both skeletal muscle\textsuperscript{733} and adipose cells\textsuperscript{734} and has consequently been shown to have a role in the insulin signalling pathway in adipocytes.\textsuperscript{735} \textit{GADD45B}, \textit{DUPS1} and \textit{TP53INP1} all have roles in regulating apoptosis and had decreased expression after RYGB surgery. \textit{GADD45B} has been shown to be induced by NF-κB during acute inflammation and is thought to be involved in repressing cell death\textsuperscript{736} and mediating protective effects on cells against DNA damage.\textsuperscript{737} \textit{DUSP1} belongs to the family of MAPK phosphatases that inactivate the MAPKs and thus negatively regulate MAPK signalling.\textsuperscript{738} It was found to be differently regulated by ER stress and had a pivotal role in ER stress-induced apoptosis.\textsuperscript{739} Even though \textit{DUSP1} null mice are resistant to diet-induced obesity, they develop glucose intolerance suggesting it has a complex role in regulating metabolic homeostasis.\textsuperscript{740}

Taken together, these data further confirm the involvement of ER stress and UPR in insulin resistance and type 2 diabetes. Furthermore, ER stress at the liver may be critical to the development of secondary peripheral insulin resistance, with hepatic insulin resistance being a primary event in the progression to type 2 diabetes.\textsuperscript{319}

### 6.6 The liver and inter organ communication

Despite the key roles of muscle and adipose tissue in insulin resistance, the liver is increasingly being recognized as the organ of prime importance in the progression to type 2 diabetes. Clinical evidence has shown that resolution of liver, but not peripheral, insulin resistance is associated with remission of type 2 diabetes after bariatric surgery,\textsuperscript{257, 448, 475-477} whereas improvement of NAFLD after a low calorie diet has been associated with normalization of fasting hyperglycaemia.\textsuperscript{256, 257} Although the molecular mechanisms behind this are unclear, several studies offer some insight. Activation of the UPR by ER stress was shown to mediate cross talk between the liver and peripheral organ insulin sensitivity via IGFB3.\textsuperscript{319} At the same time, iLIRKO mice, which develop peripheral insulin resistance in muscle tissue after primary hepatic insulin resistance due to IR ablation, also had elevated levels of IGFBP3.\textsuperscript{123}
In this study, several liver genes that code for secreted proteins had differential expression after RYGB surgery. These genes act on other insulin target tissue and have been found to regulate metabolic homeostasis and insulin secretion. This adds another dimension to the multiple lines of evidence that suggest the liver may be of prime importance in the progression to type 2 diabetes. Interestingly, two members of the IGFBP family (IGFBP2 and IGFBP5), which modulate the activity of IGF-I and IGF-II, were increased after RYGB surgery (Table 5-2). As discussed previously, IGFBP2 is increasingly being associated with regulation of metabolic homeostasis. IGFBP5, however, is a secreted protein that has been shown to have a role in promoting growth and proliferation of the pancreatic β-cell. The role the liver plays in regulating insulin release from the pancreas is of great importance considering the final event in the progression to fasting hyperglycaemia and type 2 diabetes is thought to be β-cell failure. Indeed, expression of several other genes which were found to have roles in regulating insulin secretion was also changed after RYGB surgery. Most notable of these is INHBE, which as discussed previously, may be a potential therapeutic target for preventing progression to type 2 diabetes.

The fact that INHBE is secreted by the liver and acts directly on the pancreas to regulate insulin secretion demonstrates again the central role the liver appears to have in regulating the consequences of insulin action. Another gene that may affect pancreatic β-cell size is MAP2K, expression of which was increased after RYGB surgery. Imai et al. (2008) have shown that liver ERK1/2 activation by MAP2K can induce pancreatic β-cell growth via neuronal signals from the liver. This suggests that increased liver MAP2K1 expression after RYGB may increase β-cell mass, which in turn could contribute to the observed increase in insulin sensitivity.

FNDC5 is another recently identified gene that exemplifies the central role of the liver. It was identified by Boström et al. (2012) as a hormone that is cleaved and secreted into the circulation. FNDC5 was initially found to be secreted from muscle as Irisin and early work suggested that its primary function is to induce browning of white adipose fat (WAT). Brown adipose tissue (BAT) has been found to be lower in obese individuals, while increasing the amount of BAT in rodents reduced body weight and improved glucose homeostasis.
FNDC5 is induced by exercise \(^{744,749,750}\) and has consequently been suggested to be a possible treatment for obesity and insulin resistance.\(^{751}\) However, data presented here shows that FNDC5 expression is decreased in the liver tissue of individuals who undergo RYGB surgery. This is in accord with the study of Huh et al. (2012) which showed a similar decrease in FNDC5 expression levels in muscle following bariatric surgery.\(^{750}\) Further work is needed to elucidate the exact mechanisms governing FNDC5 function and the relevance of its expression in the liver.

Evidently the liver has a critical role in inter-organ communication that leads to regulation of glucose homeostasis and secretion of insulin that is only now becoming recognised. The central role it occupies as a master regulator of metabolic homeostasis is also apparent in its regulation of circadian metabolic activity.\(^{752}\)

### 6.7 Circadian rhythm

Regulation of metabolism in the liver follows diurnal patterns that are governed by the light/dark cycle and food availability.\(^{752}\) The modern lifestyle of high caloric intake and sleep deprivation can impair the central circadian clock, but the impact it has on the molecular clock in the liver is unclear.

Nonetheless, disruption of the circadian rhythm has been documented to increase risk of obesity and diabetes.\(^{753-757}\) Interestingly, hepatic insulin sensitivity may also have a diurnal pattern. At the time of writing, a key study by Shi et al. (2013) demonstrated that insulin action has a circadian rhythm and that disrupting its rhythm increased the risk of acquiring insulin resistance and obesity.\(^{758}\) Although the link between circadian disruption and insulin resistance has been established, the molecular mechanisms governing molecular clock participation in insulin-mediated metabolism are yet to be fully identified. This study presents three potential candidate genes that are involved in the circadian rhythm and may have a role in the pathogenesis of type 2 diabetes.

Several genes involved in molecular clock regulation of metabolic homeostasis were differentially expressed after RYGB surgery. Expression of BHLHE40 and CCRN4L was decreased after RYGB surgery, whereas expression of ARNTL was increased.
All of these genes have been found to have an impact on regulation of metabolic homeostasis. BHLHE40, as discussed previously, regulates glucose metabolism through its ability to suppress CLOCK/BMAL1 enhanced promoter activity.\textsuperscript{607, 610} CCRN4L is one of a host of genes associated with control of circadian rhythm and has been implicated in regulating the metabolic processes and inflammation.\textsuperscript{759, 760} It has deadenylase activity and was shown to remove poly(A) tails from mRNA causing destabilization of mRNA and providing post transcriptional silencing.\textsuperscript{761, 762} Mice lacking CCRN4L have reduced expression of lipogenic genes and are resistant to diet-induced obesity and hepatic steatosis.\textsuperscript{763} Recently, CCRN4L from adipose tissue was shown to negatively correlate with plasma insulin levels and HOMA-IR measure of insulin resistance,\textsuperscript{764} suggesting it has a key role to play in type 2 diabetes. Decreased expression of \textit{BHLHE40} and \textit{CCRN4L} is most likely associated with the observed decreases in lipogenic gene expression and improvement of glucose homeostasis observed after RYGB surgery.

Interestingly ARNTL (alternatively know as BMAL1) is another gene involved in circadian rhythm regulation that was found to regulate \textit{CCRN4L} expression.\textsuperscript{765} Whereas \textit{CCRN4L} decreases after RYGB surgery, \textit{ARNTL} expression increased. ARNTL is a major component of the positive and negative transcriptional feed back loops that belong to the intracellular clock mechanism driving rhythmic gene expression.\textsuperscript{766} ARNTL null mice were shown to have increased levels of triglycerides, free fatty acids and cholesterol which resulted in ectopic fat formation in the liver and skeletal muscle.\textsuperscript{767} Furthermore, ARNTL knock out mice were locked into a state where insulin action was at its lowest,\textsuperscript{758} suggesting ARNTL may be a molecular link between the circadian rhythm and regulation of insulin action. The fact that it increases after RYGB surgery is consistent with this hypothesis.

\textit{BHLHE40}, \textit{CCRN4L} and \textit{ARNTL} are presented here as candidate genes that provide a link between disrupted circadian rhythm and the aberrant regulation of metabolic homeostasis seen in type 2 diabetes. Further work on the molecular mechanisms behind this process will add to our understating of type 2 diabetes and may lead to improvement in therapies that both prevent and treat type 2 diabetes.
6.8 Concluding remarks

Using the RYGB surgery as a human model to study type 2 diabetes has yielded new associations and strengthened old concepts. The pathological role lipotoxicity, inflammation, and ER stress have in type 2 diabetes was evident in the decreased expression of genes involved in these processes after remission of the disease. Evidence was also provided for the previously hypothesised link between inflammation and metabolism via the reaction of CRP, IGFB2 and leptin signalling. Furthermore, the association between the disruption of the circadian rhythm and increased risk of obesity and insulin resistance was observed in the three genes involved in regulating circadian gene expression that were differentially expressed after RYGB surgery (BHLHE40, CCRN4L, and ARNTL).

The hypothesis that the liver may be of prime importance in the progression to type 2 diabetes is supported by the differential expression of genes that code for secreted proteins that act on other insulin target tissues. Most notable of which is the liver’s ability to regulate insulin secretion by the pancreas via INHBE.

In conclusion, use of human models is a rare and priceless tool for the study of disease progression. Although use of animal models can dissect key pathways, it is difficult to design a model that substitutes for the variability of the human population in both genetic and environmental aspects. Type 2 diabetes, which was initially strongly associated with obesity, is now associated with multiple pathological process that includes lipotoxicity, inflammation, ER stress and disruption of the circadian rhythm. Data presented here identifies the liver as a key organ in the progression to type 2 diabetes and identifies several candidate genes that may have critical roles in these processes.
CHAPTER SEVEN: Final discussion and experimental limitations
Humanity is in the midst of an insidious pandemic of type 2 diabetes mellitus. Fuelled by improvements in life expectancy and living standards, the prevalence of type 2 diabetes is predicted to increase to ~552 million by the year 2030\(^1,2\) at which point it is projected to be the 7\(^{th}\) leading cause of death worldwide.\(^768\) Our understanding of type 2 diabetes pathogenesis, or lack thereof, is hindering the effective treatment of this disease. Currently there are no known pharmacological interventions that can induce remission of type 2 diabetes. Bariatric surgery, however, has been shown to cause rapid remission of type 2 diabetes and insulin resistance.\(^430, 431, 448\) This surgery presents researchers with an opportunity rarely afforded to other fields whereby tissue from human individuals can be sampled before and after remission of type 2 diabetes.

Although animal models have improved our understanding of some of the molecular processes in type 2 diabetes, they do not adequately model the complex development of metabolic syndrome and diabetes in humans. Transgenic animal models often have overexpression or complete ablation of a single gene, whereas high fat diet-induced diabetes models do not mirror the complex nature of human feeding behaviour. With this in mind, the use of the RYGB operation provides a unique opportunity to study the disease and its remission in humans and was used in this study to produce several novel findings which are summarised in the following text.

Insulin resistance is the fundamental pathology present in type 2 diabetes and has different consequences in the various insulin target tissues. The liver is responsible for a wide variety of biological process regulated by insulin which includes: cell growth, lipid and glucose homeostasis, and regulation of insulin concentrations throughout the body via hepatic insulin clearance. Unregulated hepatic gluconeogenesis is thought to be a critical component of the fasting hyperglycaemia in late stage type 2 diabetes,\(^19, 21, 23, 515\) and multiple lines of evidence suggest that hepatic insulin resistance may lead to secondary peripheral insulin resistance and glucose intolerance.\(^123, 319, 348\) Tissue specific IR knockout mice show that of the three traditional insulin target tissues involved in glucose homeostasis, the liver may be of greatest importance.\(^111, 116, 123, 516\) The liver also appears to be a vital organ in the remission of type 2 diabetes after RYGB surgery.\(^448, 476, 477, 481\)

This thesis focused on the liver and the critical role it has in regulation of metabolic homeostasis. Liver tissue was biopsied from individuals undergoing RYGB surgery.
and again from a subset of individuals at a subsequent operation. RNA and protein extracted from this liver tissue was used to investigate levels of molecules previously implicated as well as to identify novel molecular processes involved in the pathogenesis of type 2 diabetes. To achieve this, all individuals included in the study cohort were metabolically characterised and grouped according to presence of normal glucose tolerance (NGT group) or type 2 diabetes (T2DM group).

Mean fasting plasma glucose and HbA1c levels were normal in the NGT group and significantly elevated in the T2DM group (Table 2-2). Although insulin resistance was present in varying severity in all groups, the T2DM group was the most insulin resistant (Table 2-2). After RYGB surgery, however, both groups had a significant decrease in fasting plasma insulin concentrations and HOMA-IR values (Figure 2.3-2A and C). Resolution of hepatic insulin resistance was associated with remission of diabetes in 88% of individuals, which is similar to remission rates previously reported. Those individuals who had a subsequent liver biopsy (sNGT and sT2DM group), and were used to model diabetes remission, were shown to be representative of the main study population (Figures 2.3-1, 2.3-2 and 2.3-3). Importantly, there were no differences in BMI between any of the groups at any time point measured allowing us to control for confounding variables such as weight loss.

However, there are several limitations regarding the study cohort. There is no control group of individuals that did not have diabetes or RYGB surgery. This was unavoidable because of the difficulty in sampling liver tissue from human individuals. Even though we made comparisons between individuals with and without type 2 diabetes, this was done to delineate differences in diabetes and normal glucose tolerance rather than to control for experimental manipulation. Human populations are notoriously variable and even though this is one of the strengths of this thesis, it is also one of its weaknesses. Because of the relative rarity of liver tissue biopsied from the same individual before and after surgery there was little flexibility for controlling for study group demographics. Individuals with type 2 diabetes were on average older than those with normal glucose tolerance perhaps reflecting the progressive profile of this disease. Furthermore, there were a greater proportion of females in the study cohort. Although gender does effect expression of certain genes, this is unlikely to be the case with genes that are involved in metabolic homeostasis.
Another caveat is the fact that almost half of the individuals with type 2 diabetes were on some form of pharmacotherapy, which may have affected some of the gene changes observed in the microarray analysis, particularly those involved in xenobiotic metabolism. Even so, the varied therapies the individuals with type 2 diabetes were on before remission of diabetes did not appear to affect the conclusions reached in this thesis. Finally, the sample size for the individuals who had a second liver biopsy was small which presents another limitation, although this is somewhat mitigated by the fact that the data is paired, which increased the power of the study. Overall the data presented here are of considerable value to the study of type 2 diabetes.

7.1 Insulin processing at the liver: a new role for ENPP1

The liver is the first organ to receive insulin secreted from the pancreas and hepatic insulin clearance is critical in regulating the peripheral insulin concentration. Hepatic insulin clearance has been shown to increase after RYGB surgery.\(^{482}\) Data presented in Chapter 3 shows that this is not due to changes in levels of CEACAM-1 and IDE, both of which mediate the rate of insulin clearance via regulation of insulin internalization\(^{66}\) and degradation\(^{63}\) respectively.

A major finding in this study is the identification of a novel relationship between liver ENPP1 and insulin signalling at the liver. ENPP1 protein has previously been found to be increased in muscle, adipose and skin tissue of insulin-resistant individuals and its ability to inhibit insulin signalling has lead to the hypothesis that it may cause insulin resistance.\(^{167-170}\) Because of this we assayed ENPP1 mRNA expression and protein abundance in individuals with and without type 2 diabetes before and after RYGB surgery. Somewhat surprisingly, in this study, ENPP1 protein levels were lower in individuals with type 2 diabetes when compared to those individuals with normal glucose tolerance. Furthermore, those individuals who experienced remission of type 2 diabetes after RYGB surgery had an associated increase in ENPP1 protein levels.

Although this is the reverse of what has been reported previously in the literature, this and other evidence suggests that the increase in ENPP1 after remission of diabetes may be associated with its purported role as a desensitizer of insulin signalling.\(^{503}\)
Considering the liver is exposed to supraphysiological levels of insulin during first phase insulin secretion, a mechanism which prevents overstimulation of the biological pathways may be necessary for normal liver function. The role of ENPP1 as a desensitizer of insulin signalling has previously been hypothesised by Menzaghi et al. (2003) who showed that insulin stimulation promotes ENPP1 recruitment to the cell membrane where it can inhibit insulin signalling. Data presented in Chapter 3 that shows ENPP1 is inversely correlated to HOMA-IR levels further supports its role as a natural modulator of insulin signalling and suggests that the higher the insulin sensitivity the more liver ENPP1 is needed to dampen insulin signalling.

Indirect evidence also suggests that ENPP1 may be closely associated with the first phase insulin response. Individuals with type 2 diabetes have a diminished first phase insulin response, which has been shown to return to normal after gastric bypass surgery. At the same time, our data shows that ENPP1 is lower in individuals with type 2 diabetes and increases after RYGB surgery. Taken together, these data suggest that one of the roles of liver ENPP1 may be to act as a natural desensitizer of insulin signalling in response to first phase insulin secretion.

Although the work presented in this thesis suggests that ENPP1 has natural modulating action on insulin signalling strength, it cannot yet be concluded that ENPP1 has no role in insulin-resistant pathology. Indeed, the relatively rare Q121 variant has been shown to have a greater inhibitory effect on insulin receptor than the more common K121 variant and the ENPP1 K121Q polymorphism has been associated with type 2 diabetes. Further research on the role of ENPP1 in type 2 diabetes will likely focus on the frequency of the K121Q polymorphism, although data presented in this thesis suggest that ENPP1 may not be as relevant as previously thought.

There are some limitations to the study design and experimental methods used in Chapter 3. Although the technology used in this thesis has been tried and tested, in some cases better alternatives are available. Western blotting is an excellent technique to assay semi-quantitative levels of protein. However for increased accuracy and sensitivity, enzyme linked immunosorbent assay (ELISA) should have been used to assay for differences in protein concentration between study groups.
Due to funding and time constraints; however, this was not achievable. Nonetheless the relationship between liver ENPP1 protein content and hepatic insulin processing is evident with the western blot data provided.

7.2 Insulin receptor isoforms in diabetes: detrimental affects of IR-A overexpression on regulation of glucose homeostasis

Another important finding of this work is the observation that the liver IR isoform ratio is altered in type 2 diabetes and is normalised after RYGB surgery. When they were first identified the IR isoforms were quickly shown to have different functional characteristics. Multiple studies have investigated whether the IR isoform ratio is altered in muscle and fat tissue of individual with type 2 diabetes or insulin resistance, but differences in technique, and variability of study population led to conflicting results in the literature.

Early work on the functional characteristics showed that IR-A bound and internalized insulin more efficiently, whereas insulin binding to the IR-B isoform induced stronger activation of the IR tyrosine kinase. It is generally accepted that IR-A mediates mitogenic signalling (because of its association with fetal and cancer cells), and IR-B predominantly mediates metabolic signalling (because of its association with metabolically active tissue). Because of the purported metabolic signalling through the IR-B isoform, it is not surprising the liver IR-B:A ratio was found to be 9.8 in normal lean individuals. However, data reported in Chapter 4 shows that the IR B:A ratio is decreased during type 2 diabetes. Obese individuals with type 2 diabetes had a significantly lower IR-B:A ratio (5.2) than obese individuals without type 2 diabetes (6.6). In addition, the IR-B:A ratio increased from 5.4 to 8.6 only in those individuals who experienced remission of diabetes.

This change in IR-B:A ratio was caused by a significantly reduced expression of IR-A isoform, suggesting that abnormally elevated levels of IR-A may have functional consequences for insulin signalling during type 2 diabetes.
Indeed, overexpressing IR-A isoform in HepG2 cells had no affect on AKT activation and reduced insulin’s ability to inhibit \(PCK1\) transcription, whereas overexpressing IR-B caused a 50% increase in AKT activation and tended to improve insulin downregulation of \(PCK1\). Consequently it can be inferred that the abrogated IR-B:A ratio in type 2 diabetes may have a critical role in dysregulated gluconeogenesis. In accordance with our data, elevated expression of IR-A in muscle tissue of individuals with MD1, which is characterised by severe hyperinsulinaemia, has also been associated with impaired glucose homeostasis.\(^{415}\) Furthermore, because IR-A is a predominant mitogenic receptor, an increasing awareness of the link between hyperinsulinemia and carcinogenesis\(^ {428}\) provides circumstantial evidence for a relationship between hyperinsulinemia and increased IR-A expression. Although elevated levels of IR-A appear to be associated with hyperinsulinaemia, treating Hep G2 cells with supraphysiological levels of insulin did not induce changes in IR-A isoform expression (Figure 4.3-6). The stimuli that regulate alternative IR splicing are unknown, but are likely to be associated with the metabolic milieu observed in insulin resistance and type 2 diabetes.

There are several caveats with using Hep G2 cells to model normal human liver cells. Although Hep G2 cells are a well characterised liver cell line used by many investigators to model insulin signalling,\(^{506, 769-775}\) they poorly reflect the behaviour of differentiated liver tissue. Unlike normal human liver cells, which have 10 times more IR-B expression than IR-A (IR-B:A ratio of 9.8), HepG2 cells have twice as much IR-A than IR-B (IR-B:A ratio of 0.5, Figure 4.3-6). Hep G2 cells need supraphysiological concentrations of insulin to stimulate molecules such as AKT, possibly because of the much reduced IR-B:A ratio. Such high concentrations of insulin will also activate IGF-I receptors and IGF-I/INSR hybrids thus potentially confounding the conclusions made from our data. But the constant expression of IGF-IR across all transfected cells should control for this factor. Further, because Hep G2 cells originated from a hepatocellular carcinoma, it is unknown whether they kept the same mechanism by which insulin may regulate \(PCK1\) expression. However, the empty vector transfected Hep G2 cells had a significant increase in AKT phosphorylation and inhibition of \(PCK1\) transcription 5 minutes and 12 hours after
treatment with insulin respectively, which is consistent with previously reported work. To improve the experiment, a time course of AKT phosphorylation at lower insulin concentrations would have provided stronger evidence for the purported harmful effects of increased IR-A expression. Another experimental limitation is the lack of data relating to the protein levels of the two isoforms. Although an antibody to a peptide corresponding to the exon 11 of IR-B isoform was produced, it was found to be non-specific and could not be used to assay levels of IR-B protein. Nevertheless, IR isoform mRNA levels have previously been shown to mirror the relative levels of the two proteins on the cell surface.

All the caveats withstanding, the conclusions reached still have some impact on insulin’s ability to regulate metabolic signalling through the two IR isoforms. A transgenic mouse model, although technically challenging, would be useful to fully understand the effect of increased IR-A expression on insulin’s ability to regulate metabolic signalling and is an avenue for further work.

It is difficult to ascertain whether the altered liver IR-B:A ratio observed in type 2 diabetes is the cause or result of insulin resistance, but the data presented suggest that it has a role in dysregulated glucose homeostasis. Although the proteins that regulate alternative IR isoform splicing have been identified (CUG-BP1, SRp20 and SF2/ASF), the molecule or molecules that regulate their activity are still unknown. Further work should aim to elucidate the relationship between hyperinsulinaemia, the spliceosome and metabolic homeostasis. The role of the spliceosome in regulating variable signalling in different tissue and the consequences of this on metabolic homeostasis is worthy of research in and of itself. It adds another dimension to the pathogenesis of type 2 diabetes, a better understanding of which will lead to more efficient treatments of this disease. Data presented in Chapter 4 reinvigorates the role of IR isoforms in the pathogenesis of type 2 diabetes and suggests that aberrant regulation of IR isoform in metabolically active tissue has adverse affects on insulin signalling.
7.3 Changes in gene expression after remission of diabetes reveal new roles for the liver in regulating metabolic homeostasis

From the data presented so far it is clear that using RYGB surgery with a study cohort controlled for degree of insulin resistance is an excellent human model of type 2 diabetes. Nowhere is this more evident than in the microarray data presented in Chapter 5, which shows that the majority of genes that were differentially regulated after RYGB surgery are known to be implicated in the multiple pathological processes involved in type 2 diabetes. Not surprisingly, expression of genes involved in lipotoxicity, inflammation and ER stress were decreased after RYGB surgery, particularly in those individuals who had remission of type 2 diabetes.

Although there were many significant observations, two of them are of particular interest and present novel data that offer insights into the pathogenesis of type 2 diabetes. The apparent inter-organ communication between the liver and the pancreas presents a hitherto unconfirmed relationship whereby the liver can not only mediate pancreas β cell size, but can also regulate insulin secretion. *IGFB5*, which is made and secreted by the liver, is increased after RYGB surgery and has been shown to induce pancreatic cell growth.\(^742\) Similarly, expression of *MAP2K1*, which was shown to be involved in a pathway that stimulates pancreatic cell growth via neuronal signals from the liver,\(^743\) was also increased after remission of type 2 diabetes.

*INHBE*, a newly identified activin which is involved in liver-pancreas cross talk presents as a promising candidate for treatments that prevent progression from impaired glucose tolerance to type 2 diabetes. In this study, expression of liver *INHBE* tended to be higher in individuals with type 2 diabetes and decreased after RYGB surgery. Recent work on the functional characteristics of INHBE showed that it is secreted by the liver in response to insulin stimulus and acts on the pancreas to inhibit insulin secretion.\(^571-573\) Furthermore, overexpression of INHBE in mice impaired growth of pancreatic exocrine cells.\(^573\) INHBE secretion by the liver appears to be a natural feedback response to elevated levels of insulin and may contribute to the gradual loss of beta cell function that eventually precipitates diabetes. To test this hypothesis, we would measure *INHBE* protein concentrations from hepatic portal vein blood to confirm its high concentrations in individual with insulin resistance.
Following this, we would treat human pancreatic cells with INHBE protein to test what concentration of INHBE can induce β-cell failure. Finally, a transgenic animal model of insulin resistance in which we can induce INHBE overexpression would provide strong evidence for the role of INHBE in diabetes progression.

The microarray data also presented several candidate genes that are involved in circadian like regulation of metabolic homeostasis. Although disruption of the circadian rhythm is increasingly being associated with an increased risk of development of insulin resistance or type 2 diabetes,753-757 the molecular mechanism governing this link is yet to be indentified. Data presented in Chapter 5 identifies BHLHE40, CCRN4L and ARNTL as candidate genes that may be directly involved in the pathogenesis of type 2 diabetes. In particular, expression of BHLHE40 was only significantly decreased in those individuals who had a remission of type 2 diabetes. All three genes have critical roles in the molecular clock and transgenic animal models have shown them to be critical in appropriate regulation of metabolic homeostasis.606, 607, 611, 763, 767 Although the increasing association of the circadian rhythm with type 2 diabetes is relatively recent, data presented here reveals three genes that may be directly involved with insulin’s purported diurnal variation.758

However, the gene changes observed in the microarray data also suffer from the causality paradigm in that it cannot be ascertained whether the variable gene expression contributes to or is a result of type 2 diabetes. This is a common limitation of microarray analysis which is exacerbated by the multitude of genes that have no known link to type 2 diabetes or are yet to be fully characterised. For example, LGALS4 and PYROXD2 are for the first time linked to type 2 diabetes in this study. Although they may be associated with host defence and the gut microbiome, lack of functional work in this thesis prevents us from drawing any further conclusions. Microarray data can often be overwhelming, but a thorough literature search and investigation of the significant gene changes has presented multiple avenues for future research. The most promising of which would be full characterisation of INHBE’s involvement in regulation of insulin secretion and its role in the progression to type 2 diabetes.
Further, this thesis demonstrates the polygenic nature of type 2 diabetes by the array of gene changes involved in multiple pathological processes. Involvement of lipotoxicity, inflammation and ER stress has been well documented, while the importance of the gut microbiome and disruption of the circadian rhythm increased in importance with data presented here. Investigation and functional characterisation of any of the genes identified and how they interact in health and during progression to type 2 diabetes will greatly aid our understanding of this disease. Using the RYGB surgery as a model of type 2 diabetes and technologies such as exon junction arrays, whole genome sequencing, methylation and miRNA analysis will also allow for a complete picture of changes in gene expression, epigenetic and transcriptomic regulation that will eventually lead to better understanding of type 2 diabetes.

7.4 Conclusion

Type 2 diabetes is the culmination of multiple pathological processes that results from abnormal feeding, activity and circadian behaviour. To our knowledge, this is the first study that has used the RYGB surgery as a human model of type 2 diabetes to conduct a focused investigation on molecules previously implicated in the pathogenesis of said disease. Novel findings around the role of liver ENPP1 and the IR isoforms in insulin signalling have enhanced our understanding of the insulin signalling pathway and through that, the pathogenesis of type 2 diabetes. In addition, the improvement of liver insulin resistance that is associated with remission of type 2 diabetes allowed for identification of novel relationships, the most promising of which is the involvement of INHBE in type 2 diabetes progression. As with most research, this thesis provides some answers but raises even more questions. Nonetheless, the evidence presented here develops the field of type 2 diabetes research further and provides new avenues for future studies.
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APPENDIX

To avoid gel artefacts all samples to be compared were loaded onto the minimum amount of gels. Two different gels were run per antibody. For NGT vs T2DM comparisons, individuals without type 2 diabetes were loaded adjacent to individuals with type 2 diabetes. This alternating sequence was followed through the gel.

For the pre and post RYGB comparison there were six pairs of liver samples from individuals with normal glucose tolerance (sNGT) while there were seven pairs of livers from individuals with type 2 diabetes (sT2DM). Pre and post RYGB samples were loaded adjacent to each other. sNGT and sT2DM samples were alternated.

9.1 Western Blots for Chapter 3

Figure 9.1-1. ENPP1 (110-130kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.
Figure 9.1-3. IDE (118kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.

Figure 9.1-4. CEACAM-1 (160-180kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.
Figure 9.1-5. Actin (43kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.

Figure 9.1-6. ENPP1 (110-130kda) western blot using protein extracted from liver tissue biopsied from individuals both at the time of RYGB surgery and again at a subsequent operation.
Figure 9.1-7. IDE (118kd) western blot using protein extracted from liver tissue biopsied from individuals both at the time of RYGB surgery and again at a subsequent operation.

Figure 9.1-8 CEACAM-1 (160-180) western blot using protein extracted from liver tissue biopsied from individuals both at the time of RYGB surgery and again at a subsequent operation.
Figure 9.1-9 Actin-1 (43kda) western blot using protein extracted from liver tissue biopsied from individuals both at the time of RYGB surgery and again at a subsequent operation.
9.2 Western Blots for Chapter 4

Figure 9.2-1. Insulin Receptor β subunit (95kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.

Figure 9.2-2. Insulin Receptor β subunit (95kda) western blot using protein extracted from liver tissue biopsied from individuals both at the time of RYGB surgery and again at a subsequent operation.
9.2.1 AKT Phosphorylation Western Blots

Cell lysate from Hep G2 cells overexpressing IR-A or IR-B was resolved on 10% SDS-PAGE gels and transferred to PVDF membrane. After transfer the membrane was stained with Ponceau S stain and cut according to molecular weight to allow for simultaneous probing of Insulin receptor β-subunit, Actin and phosphorylated AKT. The portion of the membrane probed for p-AKT was stripped and re-probed for total AKT. Figure 6 presents all 9 biological repeats that were used to estimate p-AKT levels.

Figure 9.2-3. AKT phosphorylation in HepG2 cells overexpressing IR-A (Hep G2 IR-A) or IR-B (Hep G2 IR-B). Empty vector was used as control (Hep G2 EV).
9.3 Western Blots for Chapter 5

Figure 9.3-1. LGALS4 (36kda) western blot using protein extracted from liver tissue biopsied from individuals in the sNGT and sT2DM group at the time of RYGB surgery.

Figure 9.3-2. LGALS4 (36kda) western blot using protein extracted from liver tissue biopsied from individuals at the time of RYGB surgery.