The Role of Adipose tissue in the Pathogenesis of Non-alcoholic Steatohepatitis

Tenzin D. Dagpo
B.Sc (Hons), M.Sc

A thesis submitted for the degree of Master of Philosophy of The Australian National University
June, 2015
Statement of Originality

This thesis is submitted to Australian National University in fulfilment of the requirements of the degree of Master of Philosophy. It represents my own original work towards this research degree, except where due acknowledgements are made, and contains no material that has been previously submitted to obtain a degree or diploma at this university or any other institution.

..................

Tenzin D. Dagpo

June, 2015
Acknowledgements

“Knowledge is in the end based on acknowledgement.”

-Ludwig Wittgenstein

First and foremost, I would like to thank my main supervisor, Dr Viviane Delghingaro-Augusto, for her valuable advice, expert technical skills and enthusiasm, for directing me in the correct path at every stage. I have not met anyone else with such passion for scientific research; and it was a relief to meet another perfectionist in the lab who understood my need to label and organize everything in sight.

I would like to express my gratitude to my co-supervisor, Prof Christopher Nolan, for his vast knowledge and guidance during my research, for encouraging me to think about why I wanted to do research and about my future plans. Thank you for taking time out of your busy schedule and looking through my thesis, even on weekends.

I would also like to thank my panel member, Prof Geoff Farrell for his expertise, advice and encouragement, especially during my panel meetings. His humour made those meetings less scary and formal. Special thanks to Fahrettin Haczeyni, for teaching me all the mice harvesting techniques that I know when I first started, and for his advice and feedback as a PhD student.

This brings me to the animal techs in the TCH animal facility, Natalia, Delia and Szusza, who were always very helpful and cheerful despite being in the basement for most of the day, and took good care of my mice every day. Also thanks to senior animal tech Ayumi, who trained me in mice handling and was very patient even when I was too freaked out to touch a live mice for the first time. Thanks for giving me candy when I stayed late in the office. I would also like to acknowledge the tremendous help Dr Peter
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“You have to give yourself credit, but not too much because that would be bragging.”

-Frank McCourt
The prevalence of the metabolic disorders, type 2 diabetes (T2D) and non-alcoholic steatohepatitis (NASH) are rapidly increasing worldwide in parallel with increasing obesity rates. These obesity-related conditions are also associated with adipose tissue inflammation and dysfunction. In response to overfeeding, some individuals develop healthy obesity and are resistant to obesity-associated metabolic diseases; whereas others readily develop one or more of these disorders. The reasons for such disparity in responses to overfeeding are unclear.

Fat aussie (foz/foz) mice have a spontaneous mutation in the Alms 1 gene, causing defective appetite regulation, overfeeding and obesity. Interestingly, after 24 weeks of age on high fat (HF) diet, female Balb/c foz/foz mice develop healthy obesity; whereas NOD.B10 foz/foz mice on HF diet develop obesity and both T2D and NASH. HF-fed NOD.B10 foz/foz mice also exhibit a relative defect in adipose tissue expansion (termed adipose restriction) compared to Balb/c foz/foz mice.

We hypothesized that a primary defect in the adipose tissue response to overfeeding in the NOD.B10 foz/foz mice underlies its propensity to develop T2D and NASH, and that this defect would not be observed in diabetes and NASH-resistant Balb/c mice. The major aim of this study therefore, was to determine the role of adipose tissue in the development of T2D and NASH using NOD.B10 and Balb/c foz/foz mice.

Female NOD.B10 and Balb/c wild-type and foz/foz mice were fed on chow or HF diet over an 8 week period, and mice length, weight, body composition, adipose tissue depots and liver weights, fed-state blood glucose and plasma insulin, intraperitoneal glucose tolerance, hepatic triacylglyceride (TG) content, plasma alanine transaminase, plasma adiponectin and monocyte chemoattractant protein-1 (MCP-1) levels, as well as
the expression of genes associated with differentiation, function, inflammation and adipokine/chemokine production were measured at various time-points from 4 to 12 weeks of age.

Obesity developed in HF-fed foz/foz mice of both strains. However, only HF-fed NOD.B10 foz/foz mice developed hyperglycaemia (from 6 weeks of age), profound hyperinsulinaemia (from 8 weeks), glucose intolerance (from 5 weeks), hepatomegaly with increased TG content (from 8 weeks) and NASH (at 12 weeks of age), whereas HF-fed Balb/c foz/foz mice only developed obesity and hepatic steatosis. Adipose tissue restriction was not clearly evident by 12 weeks of age, and although changes in the majority of measured adipose tissue genes at the mRNA level were similar in HF-fed foz/foz mice of both NOD.B10 and Balb/c strains, the diabetes prone HF-fed NOD.B10 foz/foz mice had increased expression of inflammation genes (in particular MCP-1) at 12 weeks of age.

Taken together, glucose intolerance and hyperinsulinaemia precede the development of both adipose tissue inflammation and NASH in HF-fed NOD.B10 foz/foz mice. The results suggest, therefore, that adipose tissue most probably plays a secondary role in the pathogenesis of metabolic disorders associated with obesity. However, these findings do not preclude an important role of adipose inflammation and dysfunction in the progressive worsening of metabolic disorders in susceptible subjects.
Thesis-related Presentations and Awards as first author


**Tenzin Dagpo, Ainy Khan, Fahrettin Haczeyni, Geoffrey Farrell, Christopher Nolan and Viviane Delghingaro-Augusto** (2013) “Adipose tissue and non-alcoholic steatohepatitis in high fat fed *foz/foz* mice.” Australian Society of Medical Research, ACT New Investigators Forum (NIF). **Poster presentation, Abstract #20.**

**Tenzin Dagpo, Ainy Khan, Fahrettin Haczeyni, Geoffrey Farrell, Christopher Nolan and Viviane Delghingaro-Augusto** (2013) “Adipose tissue and non-alcoholic steatohepatitis in high fat fed *foz/foz* mice.” Canberra Hospital Annual Research Meeting. **Poster presentation Abstract #88.**


*Radiology Oncology private trust fund award for the best laboratory research oral presentation.*


*ASMR 2nd Runner-up award for Best Poster Presentation.*

**Thesis-related Presentations as second author**


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<th>Abbreviation</th>
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<td>4-AAP</td>
<td>4-aminoantipyrine</td>
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<td>ACP</td>
<td>ATP-citrate lyase</td>
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<td>ADP</td>
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<td>BMP</td>
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<td>bp</td>
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<td>cAMP</td>
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<td>CCL2</td>
<td>Chemokine (C-C motif) ligand 2</td>
</tr>
<tr>
<td>CCR2</td>
<td>CC chemokine receptor 2</td>
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<tr>
<td>CD11c</td>
<td>Cluster of differentiation-11c</td>
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<td>Dexamethasone</td>
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<td>Foetal bovine serum</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<td>FTO</td>
<td>Fat mass and obesity</td>
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<td>G0S2</td>
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<td>Glucokinase regulatory protein</td>
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<tr>
<td>GDM</td>
<td>Gestational Diabetes Mellitus</td>
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<td>Geomean</td>
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<td>GH</td>
<td>Growth hormone</td>
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<td>Insulin-like growth factor</td>
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<tr>
<td>IP</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ipGTT</td>
<td>Intra-peritoneal glucose tolerance test</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>IUGR</td>
<td>Intra-uterine growth restriction</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun n-terminal kinases</td>
</tr>
<tr>
<td>K+</td>
<td>Potassium</td>
</tr>
<tr>
<td>KCTD15</td>
<td>Potassium channel tetramerization domain containing-15</td>
</tr>
<tr>
<td>kDa</td>
<td>KiloDaltons</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LC-CoA</td>
<td>Long chain acyl Coenzyme A</td>
</tr>
<tr>
<td>LCFA</td>
<td>Long chain fatty acids</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LYPLAL1</td>
<td>Lysophospholipase-like protein 1</td>
</tr>
<tr>
<td>MAG</td>
<td>Monoacylglyceride</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MC4R</td>
<td>Melanocortin-4 receptor</td>
</tr>
<tr>
<td>MCE</td>
<td>Mitotic clonal expansion</td>
</tr>
<tr>
<td>MCIP</td>
<td>MCP-1-induced protein</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MDI</td>
<td>Methylisobutylxanthine, dexamethasone and insulin</td>
</tr>
<tr>
<td>MES WAT</td>
<td>Mesenteric white adipose tissue</td>
</tr>
<tr>
<td>MetS</td>
<td>Metabolic Syndrome</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulphate</td>
</tr>
<tr>
<td>MGL</td>
<td>Monoacylglycerol lipase</td>
</tr>
<tr>
<td>MHO</td>
<td>Metabolically health obesity</td>
</tr>
<tr>
<td>MIX</td>
<td>Methylisobutylxanthine</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cells</td>
</tr>
<tr>
<td>MTCH2</td>
<td>Mitochondrial carrier 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acids</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Reduced Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NAFLD</td>
<td>Non-alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NASH</td>
<td>Non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>NCAN</td>
<td>Neurocan</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acids</td>
</tr>
<tr>
<td>NEGR1</td>
<td>Neuronal growth regulator-1</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
</tr>
<tr>
<td>OAA</td>
<td>Oxaloacetate</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PAP</td>
<td>Phosphatidic acid phosphatase</td>
</tr>
<tr>
<td>PAT</td>
<td>Perilipin, ADRP and TIP47</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxylase</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PNPLA3</td>
<td>Patatin-like phospholipase domain-containing-3 gene</td>
</tr>
<tr>
<td>POV WAT</td>
<td>Peri-ovarian white adipose tissue</td>
</tr>
<tr>
<td>PP cells</td>
<td>Pancreatic polypeptide producing gamma cells</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PPP1R3B</td>
<td>Protein phosphatase 1 regulatory subunit 3B</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acids</td>
</tr>
<tr>
<td>RAGE</td>
<td>Receptor for advanced glycation end-products</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioactive Insulin Assay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPL13a</td>
<td>Ribosomal protein L13a</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Real-Time Polymerase chain reaction</td>
</tr>
<tr>
<td>SCD-1</td>
<td>Stearoyl CoenzymeA desaturase-1</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SH2B1</td>
<td>SH2B adaptor protein 1</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of cytokine signaling-3</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>Sterol response element-binding protein-1c</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>SUB WAT</td>
<td>Subcutaneous white adipose tissue</td>
</tr>
<tr>
<td>T2D</td>
<td>Type 2 Diabetes</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris, acetic acid and ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TCF7L2</td>
<td>Encoding transcription factor-7-like-2</td>
</tr>
<tr>
<td>T-CHO</td>
<td>Total cholesterol</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TG</td>
<td>Triacylglyceride</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TMEM18</td>
<td>Transmembrane protein-18</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>Tris</td>
<td>Triethanolamine</td>
</tr>
<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling protein-1</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very long density lipoprotein</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------------</td>
</tr>
<tr>
<td>Wks</td>
<td>Weeks</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
</tbody>
</table>
1. **Introduction**

1.1 **Obesity: The Global Pandemic**

1.1.1 **Epidemiology of obesity**

Obesity is a complex condition that occurs when there is abnormal or excessive accumulation of fat in the body.\(^1\) The increased rise in obesity rates have been associated with augmented caloric intake, changes in diet composition (rich in sugars and saturated fats), combined with reduced physical activity (due to modernization) and even changes in gut microbiota.\(^2, 3\) Overweight or obesity is classified by a simple anthropometric index of weight (kg) for height (m\(^2\)) called body mass index (BMI). An individual with BMI $\geq 25$ kg/m\(^2\) is classified in the overweight class, BMI $\geq 30$ kg/m\(^2\) for obesity class I, BMI $\geq 35$ kg/m\(^2\) for obesity class II and BMI $\geq 40$ kg/m\(^2\) for obesity class III.\(^4\) Due to inter-racial differences in BMI ranges, other measurements such as waist circumference and waist-to-hip ratio are also used for classification of obesity.\(^4\)

Metabolically healthy obesity (MHO) is determined by the absence of insulin resistance, major cardiovascular risk factors or metabolic syndrome (MetS).\(^2\) However, almost 50% of metabolically healthy obese individuals were found to become metabolically unhealthy obese within 10 years.\(^5\) Metabolically unhealthy obesity is that which is associated with features of the MetS and higher rates of metabolic conditions such as type 2 diabetes (T2D) and non-alcoholic steatohepatitis (NASH), as well as cardiovascular diseases.

Obesity was previously viewed as a first world problem. However, it has now developed into a global pandemic, affecting low and middle income developing nations, especially in urban cities, with 62% of the world’s obese population now living in developing countries.\(^6\) According to a recent Lancet study of the global prevalence of
overweight and obesity from 1980-2013, the worldwide overweight and obese population has increased from 857 million in 1980 to 2.1 billion in 2013, affecting 36.9% of the male and 38% of the female adult population.\textsuperscript{[6]} Obesity is also becoming increasingly prevalent in children and adolescents, with 42 million children under 5 years of age found to be overweight or obese worldwide.\textsuperscript{[6]}

The World Health Organization (WHO) estimates over 50% of European adult men and women are overweight or obese, while around 20% men and 23% women are obese.\textsuperscript{[7]} In North America, USA still has the highest prevalence of obesity (one-third men and women are obese) and account for 13% of the worldwide obese population.\textsuperscript{[6, 8]} The national medical care costs of obesity linked diseases in adults in USA have increased to $209.7 billion, taking up 20.6% of the US National health expenditure.\textsuperscript{[9]} Obesity rates in Asian countries like China and India are relatively low. However, due to their high population, China and India jointly make up 15% of the world obese population in numbers.\textsuperscript{[6]} Obesity rates in China have increased from 1993 to 2009, from 3% to 11% in men and from 5% to 10% in women.\textsuperscript{[10]} In India, around 15% of women were found to be obese or overweight.\textsuperscript{[11]}

Africa has the double burden of under-nutrition along with rising over-nutrition rates, due to migration to cities and a sedentary lifestyle. Over 42% of South African women have been found to be obese.\textsuperscript{[6]} Countries in the Middle East have also witnessed a rapid increase in obesity rates with 66% men and 71% women classified as overweight or obese in Saudi Arabia, and 74% men and 77% women overweight or obese in Kuwait.\textsuperscript{[12]} Obesity rates have also increased in Central and South Latin America with over 30% adults obese, and 70% adults overweight or obese in Mexico.\textsuperscript{[13]}
The prevalence of obesity in Australia has nearly doubled over the past two decades, making Australia one of the fattest nations in the developed world. 60% of the Australian population are classified as overweight or obese, of which 25% are obese.\cite{14} Aboriginal and Torres Strait Islander Australians were found to be 1.9 times more likely to be obese than non-indigenous Australians, with the fourth highest diabetes rates in the world.\cite{15} Furthermore, 25% of Australian children (aged between 2-16 years) are overweight or obese, of which 6% are obese.\cite{16} The economic burden of obesity and obesity-related diseases in Australia has been found to be $21 billion in direct health care and other related costs, with an additional $35.6 billion in government subsidies costs, with a total direct annual health cost of over $56 billion.\cite{17}

1.1.2 Environmental factors leading to human obesity

A sedentary lifestyle with reduced physical activity and/or increased caloric intake with diets rich in sugar or saturated fats have been implicated to contribute to the development of obesity.\cite{18-20} Saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) have been linked to the induction of inflammation and insulin resistance, and, therefore, may confer the pathogenesis of metabolic disorders associated with obesity.\cite{20, 21}

Migration studies in Pima Indians have shown that a change in physical activity and diet due to urbanization led to Pima Indians in USA being 25 kg heavier than Pima Indians in Mexico, despite the same genetic predisposition.\cite{22}

An abnormal intra-uterine environment of malnutrition or excessive nutrient supply may also play a major role in the development of metabolic conditions later in life. Fetal under-nutrition leading to intra-uterine growth restriction (IUGR) can increase
predisposition to later onset of obesity and T2D, postulated to be due to epigenetic programming for adaptation of the fetus for expected postnatal under-nutrition. Growth restricted infants were found to be seven times more likely to develop T2D by 64 years of age.\textsuperscript{[23, 24]} Gestational Diabetes Mellitus (GDM) occurs during the later stages of pregnancy when women develop insulin resistance that cannot be compensated by increased insulin secretion. Consequential hyperglycemia poses risks for the developing child, with the development of fetal macrosomia due to intra-uterine over-nutrition. This places the baby at risk of neonatal hypoglycemia, shoulder injury and breathing problems, and also increases the chances of later development of obesity and T2D.\textsuperscript{[25, 26]} Although gestational diabetes in the mother disappears after the pregnancy, it makes her susceptible to the development of T2D later in life.\textsuperscript{[25]} GDM affects at least 4.6% of pregnant women in Australia and 2-10% of pregnancies in the USA.\textsuperscript{[25]}

1.1.3 Genetic components of human obesity

Family studies have shown that obesity has 55-85% heritability for BMI.\textsuperscript{[27]} Monozygotic twin studies have shown that there was 6 times less degree of variance of weight gain and fat distribution within twin pairs, compared to between twin pairs.\textsuperscript{[28]}

Monogenic obesity arises from single gene disorders such as mutation in the leptin gene shown in \textit{ob/ob} mice or mutation in the leptin receptor in \textit{db/db} mice.\textsuperscript{[29, 30]} Since leptin plays a major role in the energy balance of the body by regulating food intake via the hypothalamus, leptin or leptin receptor mutations in mice causes excess adiposity, reduced physical activity, obesity and diabetes. In humans, rare mutations in leptin and the leptin receptor cause pituitary dysfunction and early onset of severe obesity.\textsuperscript{[31, 32]} Another single gene disorder leading to obesity is caused by mutation in the
melanocortin-4 receptor (MC4R), which regulates the effect of leptin, and mutations in the MC4R gene in mice and humans lead to morbid obesity.\cite{33, 34} Alström syndrome, caused by a point mutation in the \textit{Alms1} gene also generates the obesity phenotype among other metabolic conditions.\cite{35}

However, most obesity is polygenic and occurs due to the interaction of many susceptible genes with environmental factors. Genome-wide association studies (GWAS) have opened the doors to uncover novel loci contributing to the development of obesity, which was impossible to be detected in candidate genes studies or family studies. GWAS are based on associations between the obesity phenotype and all common variations across the entire human genome.\cite{36} Multiple loci associated with BMI and obesity have been discovered through this unbiased methodology, most notable being the fat mass and obesity (FTO) gene. The FTO gene is still considered the most robust common obesity susceptibility locus due to its high prevalence in the population, especially among Caucasians.\cite{37, 38} This gene has been found to be expressed largely in the brain and regulates energy balance by monitoring food intake.\cite{39} Overexpression of the FTO gene in mice leads to increased food intake and obesity, while inactivation of the FTO gene in mice causes post-natal growth restriction and reduced adiposity.\cite{40, 41} In humans, a homozygous R316Q point mutation in the dioxygenase-encoding FTO gene causes inactivation of FTO enzymatic activity and results in severe growth retardation and multiple malformations.\cite{42}

Meta-analyses of GWAS data sets has uncovered even more loci including transmembrane protein 18 (TMEM18), glucosamine-6-phosphate deaminase 2 (GNPDA2), potassium channel tetramerization domain containing 15 (KCTD15), SH2B adaptor protein 1 (SH2B1), mitochondrial carrier 2 (MTCH2) and neuronal growth
regulator 1 (NEGR1), which have been found to be associated with the development of obesity.\textsuperscript{[43]}

1.1.4 Health burden of human obesity

Despite widespread global calls, the rate of obesity is still increasing worldwide, which makes obesity a major public health challenge, principally in the middle income countries. Obesity has been found to cost from 0.7% to 2.8% of a country’s total health care expenditure, with obese individuals having over 30% higher medical costs compared to non-obese counterparts.\textsuperscript{[44]} According to the latest estimates by WHO, around 3.4 million adults die worldwide each year from obesity related illnesses.\textsuperscript{[6]} Individuals with high BMI are at major risk for multiple chronic diseases, including cardiovascular disease (coronary heart disease and stroke), certain type of cancers, T2D, osteoarthritis, NASH and chronic kidney disease.\textsuperscript{[45-48]} The health consequences associated with obesity includes premature death to serious chronic diseases that could reduce or impact the overall quality of life.

1.1.5 Epidemiology of Type 2 Diabetes (T2D)

T2D is a complex metabolic disorder characterized by the inability of the body to maintain adequate insulin production (β-cell dysfunction) and reduced action of insulin in target tissues (insulin resistance), resulting in elevated blood glucose levels (or hyperglycemia).\textsuperscript{[49, 50]} T2D accounts for 90-95% of all diabetic cases and can occur at any age, mostly in overweight and obese individuals.\textsuperscript{[25]} The diagnostic criteria set for
T2D includes individuals with a fasting plasma glucose level of $\geq 7.0 \text{ mmol/L}$ (or 126 mg/dL) or a random plasma glucose measurement of $\geq 11.1 \text{ mmol/L}$ (or 200 mg/dL).\[51\]

T2D has become a global epidemic with 4.6% of the world population diagnosed with the disorder, increasing from 153 million people affected in 1980 to 382 million in 2013, with numbers predicted to rise to 592 million by 2035.\[25, 52\] Diabetes caused 5.1 million deaths in 2013 in people aged between 20 to 79 years, costing $548 billion in medical expenses worldwide. One in 10 adults in the Middle East and North Africa were found to develop diabetes.\[25\] Among Asian countries, Pakistan, India, China and Japan were most affected by diabetes with an estimated 7, 31, 20 and 6 million people affected in 2000 respectively, with predictions of a dramatic increase by 2030.\[53\]

In the USA, 11.8% males and 10.8% females over 20 years of age have been diagnosed with diabetes, making it the 7th leading cause of death, due to its association with metabolic complications.\[25\] Diabetes in children and adolescents has also increased over the years due to increased childhood obesity rates affecting around 215,000 people in USA.\[54\] A recent epidemiological study found that Native Americans and African Americans had the highest prevalence of diabetes with 14.2% and 12.6% incidence rates respectively, followed by Hispanics (11.8%) and Asians (8.4%). T2D prevalence was found to be the lowest among the Caucasian population, with only 7.1% of Caucasians diagnosed with the disease.\[54\]

In Australia, approximately 2 million people were found to have prediabetes and were at a high risk of developing diabetes.\[55\] 1.7 million people are affected by diabetes and it has been predicted that around 3.3 million people will develop the disease by 2031, costing around $10.3 billion in medical costs.\[55\] Indigenous Australians are three times more likely to develop T2D than non-Indigenous Australians.\[55\]
1.1.6 Environmental and Genetic components of T2D

T2D is a complex polygenic disorder influenced by genetic and environmental factors. However, due to the multiplicity of genes contributing to T2D development in diabetes patients and related family, no predominant genetic susceptibility locus that increases predisposition to T2D development has been discovered yet.\(^\text{[56]}\)

A population-based twin study of 606 twins in the Danish Twin Register showed that genetics play an important role in T2D development, with strong links to family history.\(^\text{[56]}\) T2D has high heritability, where the lifetime risk of developing the disease is 40% in individuals with one diabetic parent, and increases to 70% if both parents are diabetic.\(^\text{[57]}\) Interestingly, having a diabetic mother has been found to increase T2D development risk more than a diabetic father.\(^\text{[57]}\) Among ethnic groups, Pima Indians and African-Americans in USA had a higher risk of developing T2D than Caucasians, with the same observation made in Indigenous and Torres Strait islanders in Australia.\(^\text{[54, 58]}\)

T2D is a multi-factorial polymorphic disorder with variants of multiple genes associated with pathogenesis. The advent of GWAS studies has identified more than 65 genetic variants that increase the risk of T2D development. Of these, at least 10 loci found are associated with β-cell dysfunction and fewer with insulin resistance or obesity. FTO gene which is associated with obesity was also discovered during the GWAS of diabetes, suggesting a close association between obesity and T2D.\(^\text{[37]}\) Among the several loci found through GWAS, TCF7L2 (encoding transcription factor-7-like-2) was identified as the highest T2D susceptibility risk locus, with the T allele of the Rs7903146c/T polymorphism linked with β-cell demise. TCF7L2 has been proposed to regulate proglucagon gene expression, and synthesis of glucagon like peptide -1 (GLP-
1) by the intestinal endocrine L-cells, which is associated with reduction of β-cell mass and function.\textsuperscript{[59]} TCF7L2 polymorphisms in non-diabetic patients was found to cause predisposition to NAFLD, whereas in individuals with NASH, it was associated with increased hepatocyte apoptosis, altered adipokine profiles and reduced β-cell function.\textsuperscript{[60]}

Individuals with mutation in candidate genes involved in glucose metabolism and insulin secretion, such as Calpain 10, PPAR-γ and GLUT2 have also been found to be at the most risk of developing T2D.\textsuperscript{[61]}

1.1.7 Epidemiology of non-alcoholic fatty liver disease (NAFLD)

NAFLD has become one of the most common chronic end-stage liver disorders worldwide due to its progression from simple steatosis to cirrhosis and liver failure. According to the 2014 Global Guidelines published by the World Gastroenterology Organization, the prevalence of NAFLD ranges from 20 to 40\% of the general population in Western countries, and from 40-90\% in the worldwide overweight/obese population of 2.1 billion people.\textsuperscript{[6, 62-64]} NAFLD has been strongly associated with MetS, T2D and obesity, where 78\% of NAFLD patients in an American epidemiological study were diagnosed with either T2D or impaired glucose tolerance (IGT).\textsuperscript{[6, 64, 65]}

Even East Asian countries like Japan, China and Korea have recently displayed an increase of 29\% in the prevalence of NAFLD.\textsuperscript{[66, 67]} In South Asia, up to 18\% of the general population in Pakistan and over 19\% of the urban population in India is affected
by NAFLD.\cite{68,69} The mechanisms by which Asians develop NAFLD are different from the people in western countries. Differences in genetic susceptibility, body fat distribution, diet and lifestyle, can cause ethnical variations in NAFLD prevalence. Epidemiological studies have also indicated that aging is another risk factor for increased susceptibility to NAFLD, although pediatric NAFLD is becoming increasingly common due to the increase in childhood obesity rates.\cite{6,63,70}

Older patients are found to be more vulnerable to NAFLD development, possibly due to the presence of more NAFLD risk factors, such as hypertension, T2D and hyperlipidemia. They are also more likely to progress to NASH compared to younger patients.\cite{63,71} Men over 20 years of age were twice more likely to be affected by NAFLD than women up to the age of 60, after which no significant gender difference was observed.\cite{63,71} In USA, NAFLD was observed to be most prevalent among the Hispanic population (45%), followed by Caucasians (33%) and African Americans (24%).\cite{72} This could be correlated to differences in genetic predisposition to unhealthy body fat distribution, obesity, insulin resistance or T2D.

In Australia, NAFLD has become the most prevalent liver disease with 40% of adults over 60 years of age developing NAFLD.\cite{73} In 2012, 5.5 million Australians were affected by NAFLD, with a prediction of 7.2 million NAFLD affected persons by 2030. Fifteen % of Australian school children develop NAFLD.\cite{74,75} NAFLD increases the risk of liver cancer, with NSW witnessing an annual increase of liver cancer by 5.3% in men and 8.8% in women from 1997 to 2006.\cite{76} In general, around 2000 Australians die due to NAFLD and related diseases each year. Chronic liver diseases like NAFLD and hepatitis have been found to have a total burden of $50.7 billion in the Australia health system.\cite{75}
1.1.8 Environmental and Genetic components of NAFLD

GWAS have identified the rs738409, I148M allele of the patatin-like phospholipase domain-containing 3 gene (PNPLA3) as an important genetic contributor to NAFLD.[77] The PNPLA3 gene encodes for adiponutrin, an enzyme expressed in the liver and adipose tissue, responsible for the regulation of the hydrolysis of TG.[78] Homozygous carriers of this allele had double the hepatic fat content compared to non-carriers, and this allele was most prevalent in Hispanics and less in African-Americans.[79] Another variant of the PNPLA3 gene, PNPLA3-S453I, was found to be associated with low hepatic fat content and was most common in African-Americans and rare in Hispanics.[77] This suggests that the difference in NAFLD prevalence between different ethnicities could be due to variations in the genetic sequence of this gene. Homozygosity in the polymorphisms of the PNPLA3 gene has also been found to influence liver fibrosis in NAFLD patients and could determine NAFLD severity.[80]

Other loci identified through meta-analysis of GWAS data were genes encoding neurocan (NCAN; rs2228603), protein phosphatase 1 regulatory subunit 3B (PPP1R3B; rs 4240624), glucokinase regulatory protein (GCKR; rs780094) and lysophospholipase-like protein 1 (LYPLAL1; rs12137855), which have been associated with hepatic steatosis, lobular inflammation, altered serum lipid levels and fibrosis.[81]
1.1.9 Association between Obesity, T2D and NASH

A strong association between diabetes and obesity has been extensively described. Recently, the term “diabesity” was coined to describe this close relationship. Observational studies of young men with no family history of diabetes on excessive calorie intake for 6 months showed that they displayed an increase in BMI, with reversible increases in fasting glucose, insulin and triacylglyceride levels.\cite{82}

Around 90\% of people who develop T2D are obese or overweight suggesting that weight gain increases the risk of diabetes. High BMI and visceral adiposity are strongly associated with NAFLD and NASH.\cite{48, 83} T2D and NASH often co-exist during obesity, where 69\% of T2D patients develop NAFLD and the prevalence of NAFLD was found to be 64\% in South Asian subjects with T2D, while it was only 20\% in South Asians with normal glucose tolerance.\cite{64, 84} In addition, NAFLD disease progression to NASH is more common in diabetic NAFLD patients when compared to non-diabetic NAFLD patients.\cite{64} Obesity, insulin resistance and hyperinsulinemia are common entities for both T2D and NASH.\cite{85, 86}

Although there is good evidence for associations between obesity, T2D and NASH, the triggering mechanisms leading to NASH progression are still unknown. Exercise and diet weight loss intervention programs leading to reduction in obesity have shown improved blood glucose levels in diabetic patients and reduction in serum ALT levels in NAFLD patients.\cite{87} Donnelly et al also observed that most of the lipids in the liver during NAFLD originated mostly from fatty acids from peripheral adipose tissue, suggesting that adipose tissue dysfunction may be causing hepatic steatosis during NAFLD.\cite{88}
However, in the study conducted by Speliotes et al, it was suggested that fatty liver occurs due to dyslipidemia and abnormal glycemic levels, independent of visceral fat.\cite{89} Excessive fuel intake has also been associated with the development of insulin resistance and β-cell failure in susceptible cells (due to genetic or intra-uterine environmental factors), consequently leading to dyslipidemia and hyperglycemia.\cite{90} A disturbance in circulating glucose and lipid homeostasis due to islet β-cell failure could affect peripheral tissues, like adipose tissue, as well as liver, causing pathological changes such as steatosis and inflammation, which are observed during NASH and adipose tissue dysfunction (Figure 1).\cite{91}

Failure of subcutaneous adipose tissue expansion to accommodate the excess nutrients and abnormal expansion of visceral adipose tissue, which causes hypoadiponectinemia and systemic inflammation, could be implicated in the pathogenesis of NASH.\cite{85, 92} Adipose tissue dysfunction modulates the risk of liver injury and signals progression from hepatic steatosis to NASH, while pro-inflammatory adipose tissue hormones like tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) can cause inflammation and macrophage infiltration in the liver causing NASH.\cite{93, 94} Taken together, this suggests that the adipose tissue could also play a primary role in the pathogenesis of NASH.
Figure 1: Potential interplay between pathogenic factors causing T2D and NAFLD/NASH. (Nolan et al, 2010)\(^{(91)}\).
1.2 Obesity-related diseases

1.2.1 Obesity-related Type 2 diabetes (T2D)

1.2.1.1 Pancreatic islet architecture

The pancreas is a glandular organ with both exocrine and endocrine functions. Endocrine cells only populate 2% of the entire pancreatic cell mass and are arranged in small clusters of cells called islets or islets of Langerhans.[95] Islets are comprised of five main cell types: glucagon producing alpha cells (α-cells), insulin producing beta cells (β-cells), somatostatin producing delta cells (δ-cells), pancreatic polypeptide producing gamma cells (PP-cells) and ghrelin producing cells (ε-cells).[96, 97] Human β-cells are aligned along blood vessels and make up 55% of the islet, while rodent β-cells appear in clusters, making up 77% of the islet cells.[98, 99] β-cells maintain glucose homeostasis by producing and releasing insulin into the circulation.

Insulin comprises of two amino acid side chains linked together by two disulphide bonds. Insulin mRNA translates into a single chain inactive precursor called preproinsulin, which is proteolytically excised in the endoplasmic reticulum (ER) and undergoes post-translational processing to generate proinsulin. Proinsulin consists of a B chain with an amino terminal, an A chain with a carboxy terminal and a connecting C-peptide in the middle. Several specific endopeptidases in the ER cleave C-peptide to generate mature and active insulin. This insulin and C-peptide are then packaged in golgi bodies and stored in secretory granules within the cytoplasm. Stimulation of β-cells to secrete insulin leads to the release of insulin from the granules through exocytosis into circulation via the portal system.[100, 101]
1.2.1.2 Glucose-stimulated insulin secretion (GSIS)

In normal pancreatic β-cells, insulin secretion can be stimulated by several factors, including fuels such as glucose, amino acids and fatty acids, as well as non-fuel stimuli like neurotransmitters (acetylcholine), hormones and enteric factors (glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP)). The latter stimuli are known to potentiate nutrient-stimulated insulin secretion. Glucose-stimulated insulin secretion (GSIS) is mediated by two pathways: the triggering or $K_{ATP}$ dependent pathway (also known as classical pathway) and the amplifying or $K_{ATP}$ independent pathway.[102, 103]

Elevation in blood glucose concentrations results in facilitated diffusion of glucose across the β-cell plasma membrane via the glucose transporter (GLUT2 in rodents, GLUT1 in humans).[104, 105] Glucose undergoes glycolysis and generates pyruvate, which enters the Krebs cycle and generates ATP. An increase in ATP:ADP ratio causes closure of $K_{ATP}$ dependent channels in the plasma membrane, preventing $K^+$ ions flux across the membrane. Increase in intracellular $K^+$ ion concentration creates a positive charge, leading to plasma membrane depolarization. This causes the opening of the voltage-gated calcium (Ca$^{2+}$) channels, and consequent influx of extracellular Ca$^{2+}$ ions. Increase in intracellular Ca$^{2+}$ concentration triggers insulin granules exocytosis into circulation.[102, 106-108]

Signals generated by the $K_{ATP}$ dependent pathway are not enough to sustain insulin secretion. Thus, other metabolic coupling factors generated through glucose metabolism have been proposed as possible amplification pathways, such as the anaplerosis pathways, citrate cataplerosis and malonyl-CoA/LC-CoA pathway. In the anaplerosis pathways, glucose is catalyzed by pyruvate carboxylase to generate increased levels of
Krebs cycle intermediates such as NADPH and Malonyl-CoA from the malate-pyruvate shuttle. NADPH generated from the malate-pyruvate shuttle has been known to inhibit voltage-gated K⁺ channels and modulate GSIS. Malonyl-CoA blocks fatty acid oxidation and induces fatty acid esterification by inhibiting the action of carnitine palmitoyltransferase-1 (CPT-1), leading to accumulation of LC-CoA, TG, DAG and phospholipids in the cytosol. This causes accumulation of fatty acids and LC-CoA in the cytosol, leading to enhanced insulin secretion, since free fatty acids have been known to augment GSIS via K⁺ ATP independent pathways.[106-111]

1.2.1.3 Effect of insulin on peripheral tissues

Insulin is a pleiotropic hormone responsible for maintaining fuel homeostasis by regulating metabolic processes in tissues such as liver, skeletal muscle and adipose tissue.

(i) Effect of insulin on liver

Insulin activates the intrinsic tyrosine kinase activity of the β-subunits of the insulin receptor which in turn phosphorylates proximal substrate proteins like insulin receptor substrate-1 (IRS-1).[112, 113] Tyrosine kinase activation of IRS-1 initiates a signaling cascade which branches into the phosphatidylinositol-3-kinase (PI3K)-AKT pathway and the Ras-mitogen activated kinase (MAPK) pathway.[113, 114] In the hepatocytes, the AKT pathway increases glycogen synthesis by inactivating glycogenolysis. Insulin promotes glycogenesis and inhibits gluconeogenesis and glycogenolysis by regulating the activity of enzymes involved in these processes. Glycolysis stops when circulating insulin levels are low, and glucagon stimulates glycogen breakdown instead.[115, 116]
Insulin promotes the synthesis and storage of lipids in hepatocytes and inhibits fatty acid oxidation. It enhances the activity of ATP-citrate lyase, which catalyzes cytosolic citrate into acetyl CoA and oxaloacetate (OAA) during lipogenesis.\[117\] Insulin also stimulates the expression of lipogenic genes such as acetyl CoA carboxylase and fatty acid synthase that are involved in the conversion of acetyl CoA into acyl CoA, which can be converted into triacylglycerides (TG) and stored in lipid droplets.\[118, 119\] In rodents, liver-specific insulin receptor knockout has been found to lead to severe insulin resistance, glucose intolerance and failure of insulin to inhibit hepatic glucose production and progressive hepatic dysfunction.\[120\]

(ii) Effect of insulin on skeletal muscles

High blood glucose levels activate insulin secretion, which in turn promotes glucose uptake in skeletal muscles through glucose transporter GLUT4. GLUT4 is present in cytoplasmic vesicles as preformed transporters that translocate to the plasma membrane upon insulin receptor activation. When the insulin receptor is inactive, GLUT4 returns to the cytoplasmic vesicles.\[121\]

Similar to hepatocytes, in skeletal muscles, the insulin-activated signaling cascade stimulates glycogen synthesis and storage. It also promotes glycolysis. Insulin increases the storage of circulating fatty acids as TG in myocytes, which is used during exercise and for heat production.\[116, 122, 123\] Similar to insulin, exercise causes GLUT4 recruitment and increased glucose oxidation in skeletal muscles, causing up-regulation of glucose uptake.\[124, 125\] Skeletal muscle specific disruption of the insulin receptor gene in mice leads to elevated serum TG and free fatty acid levels and increases fat mass, but normal blood glucose and insulin levels are maintained, suggesting that insulin
resistance in muscle plays a secondary role in disruption of glucose homeostasis compared to other tissues such as liver and adipose tissue.\textsuperscript{[126]}

(iii) Effect of insulin on adipose tissue

Similar to skeletal muscle, glucose uptake into adipocytes is facilitated by the insulin regulated GLUT4 transporter. GLUT4 expression in adipose tissue is reduced during obesity and T2D. Mice with adipose specific GLUT4 reduction have normal growth and adipose tissue mass, but develop glucose intolerance and hyperinsulinemia.\textsuperscript{[127]}

Unlike in liver and skeletal muscles, insulin promotes the storage of glucose in the adipocytes in the form of TG, instead of glycogen, by stimulating the action of pyruvate dehydrogenase (PDH) and acetyl CoA carboxylase (ACC).\textsuperscript{[128, 129]} Insulin also stimulates the translocation of lipoprotein lipase (LPL) to the endothelial cells, where it hydrolyzes circulating chylomicron and VLDL TG to generate glycerol and fatty acids, which then enters the adipocytes to form TG.\textsuperscript{[130]} Insulin prevents the lipolysis of the TG stored in the lipid droplets by inhibiting the action of hormone sensitive lipase (HSL), which is one of the main enzymes responsible for the lipolysis of TG into diacylglycerols and monoacylglycerols.\textsuperscript{[131]}

Adipocyte specific disruption of the insulin receptor gene in mice leads to low fat mass and protection against hypothalamic lesion-induced obesity and obesity-related glucose intolerance, due to failure of insulin to induce fat accumulation. However, the adipocytes of these mice exhibited a decrease in expression of adipogenic genes such as fatty acid synthase (FAS), CCAAT/enhancer binding protein-\(\alpha\) (C/EBP-\(\alpha\)) and sterol response element-binding protein-1c (SREBP-1c).\textsuperscript{[132]}
1.2.1.4 β-cell compensation

Islet β-cells compensate with enhanced insulin secretion in response to obesity-related insulin resistance, in order to maintain euglycemia, through two mechanisms: β-cell mass expansion and β-cell function enhancement.[133, 134]

Compensatory increase in β-cell mass occurs predominantly by increase in β-cell number by replication and possibly reduced apoptosis. Some of the factors responsible for stimulating β-cell mass expansion are short-term increase in circulating glucose and fatty acid levels, enhanced activation of IRS2 by insulin and insulin like growth factors (IGF) and increase in incretin hormones like GLP-1 which stimulate Pdx1 expression, promote β-cell proliferation and reduce apoptosis.[90, 135-137] In response to insulin resistance, islets enhance β-cell function and insulin secretion by increasing glucose metabolism via up-regulated GLUT2 activity and up-regulation of both the K<sub>ATP</sub> dependent and independent pathways.[138, 139] In addition, increased expression of GLP-1 can potentiate GSIS along with promoting cell growth.[140] Both glucose and GLP-1 can also promote the transcription of the insulin gene to increase insulin biosynthesis.[141] Individuals unable to adequately compensate for insulin resistance by either mechanism are predisposed to develop diabetes.[134, 135, 138, 141, 142]
1.2.1.5 Pathophysiology of T2D

Over-nutrition and obesity have been strongly associated with insulin resistance, where a chronic excess supply of glucose and fatty acids in the blood can lead to a continuous increased demand for insulin secretion.\textsuperscript{[143, 144]} Insulin resistance develops when target tissues generate a subnormal biological response to a normal concentration of insulin.\textsuperscript{[143, 145]} Loss of insulin action could also stem from defects in the insulin signaling cascade or the increased activity of pro-inflammatory cytokines like TNF-\(\alpha\) in the adipose tissue, which has been known to decrease insulin sensitivity. Expression of insulin receptor substrate (IRS) and insulin receptors were found to be reduced in rodents and humans with T2D.\textsuperscript{[145, 146]} However, insulin resistance alone does not cause or aggravate diabetes, unless the \(\beta\)-cells are susceptible to dysfunction as well.

T2D develops when the islet \(\beta\)-cell is unable to adequately sustain the compensatory mechanisms of increased \(\beta\)-cell mass and/or enhanced \(\beta\)-cell function to increase insulin secretion, in response to insulin resistance.\textsuperscript{[100, 143]} Increase in \(\beta\)-cell apoptosis and/or reduction in cell regeneration decreases \(\beta\)-cell mass and causes \(\beta\)-cell failure, leading to T2D development.\textsuperscript{[142, 147]} Decrease in \(\beta\)-cell compensation due to loss of \(\beta\)-cell mass could lead to T2D since a 38\% reduction in \(\beta\)-cell mass was observed during autopsy of T2D patients of European origin, compared to matched controls.\textsuperscript{[148]} Loss of \(\beta\)-cell mass and consequential abnormal insulin secretion in T2D has been observed to occur when high levels of circulating glucose and fatty acid levels co-exist (known as glucolipotoxicity). Reduction in \(\beta\)-cell mass has been described during islet inflammation augmented by reactive oxygen species (ROS) or endoplasmic reticulum (ER) stress.\textsuperscript{[149, 150]}
1.2.2 Obesity-related non-alcoholic steatohepatitis (NASH)

Liver is a pleiotropic organ with critical roles in carbohydrate and lipid metabolism, synthesis of plasma proteins and drug detoxification. The liver plays an important role in maintenance of glucose homeostasis in a non-diabetic state through uptake of excess glucose levels during post-prandial states or by production of glucose during fasting state.\cite{151} In the last few decades, the Westernized lifestyle (increased food intake and reduced physical activity) leading to increased prevalence of obesity and MetS have promoted pathophysiological changes in the liver. Although adipose tissue stores circulating free fatty acids in lipid droplets within adipocytes, chronic excess nutrient supply can lead to ectopic deposition of excess fatty acids into the liver in the form of triacylglyceride (TG) leading to steatosis and lipotoxicity, possibly progressing to cirrhosis and hepatocellular cancer.\cite{152}

1.2.2.1 Liver architecture

The liver is divided by fissures into four distinct lobes: the left, right, caudate and quadrate lobes. Each lobe is formed by thousands of hexagonal lobules, which contains a central vein surrounded by six hepatic portal veins and six hepatic arteries; all connected by a fenestrated net of capillaries called sinusoids.\cite{153} The hepatic lobules are made up of three main cell types: cholangiocytes, kupffer cells and hepatocytes. Cholangiocytes form bile ducts to transport bile and maintain the pH and secretion of bile.\cite{154} Kupffer cells are macrophage cells responsible for innate immune responses and host defense in the liver, detoxification of blood from bacteria, viruses, parasites, endotoxins, alcohol and drugs, as well as the breakdown of old erythrocytes into bilirubin, a component of bile.\cite{155} In addition, Kupffer cells have also been found to
trigger early phase NASH development in a diet-induced obese mouse model, by increasing the production of tumor necrosis factor-α (TNF-α) and leading to recruitment of monocyte chemoattractant protein-1 (MCP-1). Hepatocytes make up 80% of the liver parenchyme and perform the most crucial metabolic functions of the liver, including hepatic glucose utilization and hepatic glucose production.

### 1.2.2.2 Normal hepatocyte biology

#### (i) Glucose metabolism in hepatocytes

Circulating glucose rapidly enters hepatocytes through GLUT2, a bidirectional transmembrane glucose transporter which functions independent of insulin regulation. Hepatocytes rapidly take up excess circulating post-prandial glucose. Glucokinase phosphorylates the absorbed glucose to glucose-6-phosphate (G6P), trapping it within the hepatocytes, since G6P cannot be transported out through GLUT2. Glycogen synthase then synthesizes glycogen from G6P (*glycogenesis*) and also produces pyruvate (*glycolysis*) to generate mitochondrial ATP via the Krebs cycle. Low blood glucose levels during short term fasting state activate glycogen phosphorylase to depolymerize glycogen back to glucose (*glycogenolysis*), which is released into circulation. However, if hepatic glycogen reserves deplete, hepatocytes synthesize glucose from amino acids, glycerol and non-hexose carbohydrates like lactate and pyruvate (*gluconeogenesis*).

Amino acids undergo deamination into α-ketoacids, which are converted to precursors of the Krebs cycle. Lactate and pyruvate enter the Krebs cycle to produce oxaloacetate (OAA), which is released from the mitochondria in the form of malate, and reverts back to OAA once in the cytoplasm. Phosphoenolpyruvate carboxylase
(PEPCK) catalyzes the conversion of OAA into phosphoenolpyruvate (PEP), which undergoes various phosphorylation cycles to produce fructose-6-phosphate (F6P). F6P is finally converted to G6P and dephosphorylated to generate hepatic glucose in the endoplasmic reticulum. Murine studies have shown that PEPCK null livers cause decrease in whole body gluconeogenesis from PEP, but maintain euglycemia during the fasting state by gluconeogenesis from glycerol.[159] Inhibition of the Krebs cycle leads to accumulation of Krebs cycle intermediates which increases hepatic steatosis.[160] Gluconeogenesis is regulated by pancreatic hormones insulin (inhibits) and glucagon (stimulates), growth hormone (GH) which stimulates PEPCK expression through JAK2/STAT5 pathway and gastrointestinal molecules like FGF19 and glucagon-like peptide 1 (GLP-1).[161-163]

(ii) Lipid metabolism in hepatocytes

TG present in circulating chylomicrons and adipose tissue, upon contact with lipase enzymes, is hydrolyzed into free fatty acids (FFA) and glycerol. FFAs are transported into the hepatocytes via membrane-bound transport protein fatty acid translocase (FAT)/CD36 and fatty acid transport proteins (FATP). FFAs that enter hepatocytes either undergo oxidation to generate energy and ketone bodies, or are converted to TG to be stored in lipid droplets inside the hepatocytes or exported as very low density lipoproteins (VLDL) into circulation.

During low blood glucose levels (fasting state), β-oxidation of short, medium and long chain fatty acids (LCFA) occurs in the mitochondria, while very long chain fatty acids are metabolized in the peroxisomes. Mitochondrial β-oxidation produces acetyl-CoA and generates 14 ATP of energy per oxidation cycle via the electron transport chain.[164]
Peroxisome proliferator-activated receptor-α (PPAR-α) has been identified as the main regulator of fatty acid β-oxidation. PPAR-α agonists stimulate mitochondrial β-oxidation in liver and skeletal muscles while PPAR-α nullizygous mice displayed reduced constitutive mitochondrial fatty acid β-oxidation. Peroxisomal α-oxidation and microsomal ω-oxidation produce H$_2$O$_2$ instead of NAD$^+$, and less ATP since peroxisomes do not have an electron transport chain like mitochondria. Peroxisomal and microsomal oxidation function with the mitochondria to maintain lipid homeostasis, although increased peroxisomal oxidation produces more reactive oxidation species causing hepatic oxidative stress.

Hepatocytes are capable of de novo lipogenesis by metabolizing pyruvate into citrate via the Krebs cycle. Citrate is then exported into the cytoplasm and ATP-citrate lyase (ACP) cleaves it into acetyl-CoA and OAA. Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) convert acetyl-CoA first into malonyl-CoA and then into palmitic acid, respectively. Palmitic acid forms mono- and poly-unsaturated LCFA by the action of fatty acyl-CoA elongase 6 (EVOVL6) and stearoyl-CoA desaturase-1 (SCD-1). De novo lipogenesis only makes a minor contribution to the total hepatic lipid pool. The expression of lipogenic genes like FAS, ACC and SCD-1 are promoted by insulin-stimulated SREBP1c, and glucose-activated carbohydrate response element binding protein (ChREBP).

(iii) Protein metabolism in hepatocytes

Hepatocytes are also the site for deamination and transamination of amino acids, by enzymes such as aspartate aminotransferase (AST) and alanine transaminase (ALT) which are used as clinical markers for liver damage. Hepatocytes also synthesize
most of the circulating plasma proteins, most importantly albumin, prothrombin and fibrinogen. Albumin is necessary for the maintenance of plasma osmotic pressure and transport of drugs, hormones and metabolites, whereas prothrombin and fibrinogen are important components of the coagulation system.\textsuperscript{174}

\subsection*{1.2.2.3 Pathophysiology of NAFLD}

NAFLD encompasses a wide pathological spectrum ranging from simple steatosis, to fibrosis and cirrhosis, in the absence of significant alcohol intake (<20 g ethanol/day in women, <30 g ethanol/day in men) (Figure 2).\textsuperscript{62} Simple steatosis is characterized by excessive fat accumulation in the liver in the form of TG, without inflammation or hepatocellular injury. This occurs due to altered hepatic lipid metabolism derived from an imbalance between lipid intake and production (fatty acid uptake, \textit{de novo} lipogenesis) and lipid output (mitochondrial fatty acid \(\beta\)-oxidation and lipoprotein synthesis and export) in the hepatocytes.\textsuperscript{92} Simple steatosis is a benign condition and does not cause major hepatocellular injury, unless it progresses to NASH, which is the more severe form of NAFLD. Several studies have shown that around one-third of NAFLD patients progress to develop NASH, with reduction in survival rate due to cardiovascular disorders and other liver-related complications.\textsuperscript{175, 176}

NASH is characterized by steatosis in more than 5\% of hepatocytes, accompanied by lobular and portal inflammation (macrophage and lymphocyte inflammation), hepatocyte injury (ballooning degeneration of parenchymal hepatocytes and the presence of Mallory bodies), and even hepatic fibrosis.\textsuperscript{92} NASH may remain asymptomatic for years in most cases, but up to one-third of NASH patients develop cirrhosis, ultimately leading to liver failure and/or liver cancer (hepatocellular
carcinoma) due to the destruction of hepatocytes by inflammatory cells (hepatocellular necrosis).[177]

Despite various efforts, the exact mechanism involved in NAFLD progression to NASH is still unclear. Day and James proposed the “two-hit” theory to explain the pathogenesis of NASH. According to this theory, the “first hit” is the development of hepatic lipid accumulation (simple steatosis), which is not enough to cause NASH development. But it renders the liver more vulnerable to other cellular stresses (2nd hit), causing further pathogenic processes characteristic of NASH, which include inflammation, fibrosis and hepatocellular apoptosis. Of note, several factors have been suggested to play a role as the 2nd hit in NASH pathogenesis, including pro-oxidant agents initiating hepatic lipid peroxisomal α-oxidation and microsomal ω-oxidation with the production of reactive oxygen species (ROS), circulating pro-inflammatory cytokines which may originate in inflamed adipose tissue, and excess exposure of liver to gut-derived bacterial endotoxins.[168, 178, 179]

However, this theory has now been modified to a more globally recognized “multi-hit” hypothesis; where the “first hit” has been identified to be insulin resistance with its associated factors of hyperglycemia, hyperlipidemia and hyperinsulinemia, which cause hepatic steatosis. This leaves the liver susceptible to “multiple hits” of oxidative stress from increased production of ROS, altered adipokines such as reduced adiponectin and increased TNF-α, endoplasmic reticulum stress, apoptosis and/or overexpression of Toll like receptors (TLR).[180] These “multiple hits” are different in each individual, making it difficult to identify a common established cause of NASH pathogenesis among the general population.
Figure 2: Representation of the pathological spectrum of NAFLD ranging from simple steatosis to non-alcoholic steatohepatitis (NASH) and finally ending with cirrhosis and hepatocellular carcinoma.
1.2.3 Obesity-related adipose tissue dysfunction and inflammation

1.2.3.1 Adipose tissue architecture

Adipose tissue is a loose connective tissue consisting of a heterogeneous cell population constituted by mature adipocytes (fat cells), supported by a framework of stromal cells which includes preadipocytes, fibroblasts, mesenchymal stem cells, endothelial cells and immune cells (in particular, adipose tissue macrophages). White adipose tissue (WAT) forms the majority of the adipose tissue mass and is located in large subcutaneous and visceral depots throughout the body, whereas brown adipose tissue (BAT) has limited distribution, particularly in adult individuals.\(^{[181]}\)

(i) White adipose tissue (WAT)

WAT is located throughout the body in the connective tissues underlying the skin (subcutaneous), between muscles and relates to many internal organs in the body (visceral). Each depot has its distinct morphology, developmental time and function.

In rodents, subcutaneous WAT develops before the visceral (peri-ovarian, mesenteric) WAT. White adipocytes generally have a unilocular appearance with a single large lipid droplet occupying most of the cell, pushing the cytoplasm and nuclei to the periphery.\(^{[182]}\) Subcutaneous adipocytes are heterogeneous with mature unilocular adipocytes interposed with small multilocular adipocytes, whereas visceral adipocytes are more homogenous with mostly unilocular adipocytes.\(^{[152]}\)

Subcutaneous adipocytes have a higher adipocyte turnover rate to allow enhanced storage of large amounts of TG through increased adipocyte differentiation and cell size expansion.\(^{[183]}\) This reduces lipid accumulation in visceral adipocytes and ectopic
deposition in liver and muscles. Subcutaneous adipocytes also respond more to estrogen, while visceral adipocytes are stimulated by glucocorticoids. This explains why pre-menopausal women are more predisposed to increased subcutaneous adipose tissue expansion. Subcutaneous adipocytes also express higher levels of leptin, adiponectin and glycogen synthase, while visceral adipocytes express increased levels of pro-inflammatory adipokines such as IL-6. Therefore subcutaneous adipocytes may play a protective role against inflammation. Abnormal lipid distribution into visceral adipose tissue leading to visceral or central obesity has been positively associated with insulin resistance and increased lipolysis and inflammatory cell infiltration.

(ii) Brown adipose tissue (BAT)

Brown adipose tissue is distinct from white adipose tissue in morphology, biological function and developmental time. During human gestation, BAT development is earlier than WAT and makes up 5% of the body weight in infants, which then regresses with age and is replaced by WAT instead. Interestingly, recent studies using integrated \(^{18}\)F-fluorodeoxyglucose positron emission tomography and computed tomography (PET-CT) scans have found major depots of metabolically active fat in the cervical-supravicular region of adult humans. BAT was found to be prevalent predominantly in females. This observation suggests that BAT may potentially play a greater role in human metabolism than previously described. BAT is prominent in small mammals living in cold environments and in hibernating animals. In adult mice, it is present as two large lobulated brown depots on the dorsal region of the thorax, between the scapulae.
Brown adipocytes are smaller and fewer in number and are characterized by multi-locular lipid droplets of varying sizes with considerable cytoplasm volume and single central round nuclei.[152] They also have high mitochondrial content, giving it its characteristic brown color, and unlike white adipocytes, they express mitochondrial uncoupling protein-1 (UCP-1), which allows the mitochondria to uncouple oxidative phosphorylation in the electron transport chain to generate heat instead of ATP.[192] BAT converts nutrients into heat to provide non-shivering thermogenesis to aid newborns in adapting to a lower external temperature.[193] BAT mass has been found to be inversely correlated to BMI, especially in ageing humans and has also been shown to protect mice from diet-induced obesity. [186, 188, 194-197] Therefore, beside cold-induced thermogenesis, BAT may possibly play a therapeutic role in protection from diet-induced obesity and diabetes.

Increasing interest has been on beige or brite adipocytes, brown-like adipocytes present within WAT, inducible by cold or catecholamine stimuli. In mice, beige adipocytes develop after brown adipocytes, but regress into WAT. Unlike brown adipocytes, beige adipocytes originate from non-Myf5 stem cells. Unlike white adipocytes, they express low levels of UCP-1 which is activated with cAMP stimulation. The genetic expression pattern of beige adipocytes is different from both white and brown adipocytes.[198, 199]

### 1.2.3.2 Adipocytes: Origin and differentiation

Adipose tissue is responsible for the storage and accumulation of fat in the body. Excessive nutrient supply in healthy individuals is stored in adipocytes as TG and very little accumulates in other tissues such as liver, skeletal muscles and pancreas. However, in obese unhealthy individuals (metabolically unhealthy obesity), adipocytes are unable to accommodate all the excess fats, consequently leading to spillover of TG into other
tissues which can cause tissue dysfunction. Obesity leads to adipose tissue expansion, which can occur via an increase in number of adipocytes (hyperplasia) and increase in size (hypertrophy) as well.[200]

The process where fibroblast-like preadipocytes differentiate into mature lipid-laden adipocytes, able to store fat, is known as adipogenesis. Adipogenesis is a complex multi-step dynamic process regulated by various transcription factors and cell cycle proteins, comprising of 3 distinct phases: preadipocyte determination, growth of preadipocytes and terminal differentiation (Figure 3). Adipogenesis can produce two different cell types: white adipose tissue (WAT) and brown adipose tissue (BAT), as previously discussed.

1.2.3.2.1 Preadipocyte determination phase

In the determination phase, mesenchymal stem cells (MSC) are converted to morphologically similar committed preadipocytes, which then can differentiate into adipocytes only.[201] This phase has been mostly studied in pluripotent fibroblast cell lines like C3H10T1/2 (10T1/2) cells and from adipose derived stem cells (ADSC). ADSC have been purified from the stromal vascular fraction (SVF) of the adipose tissue and can differentiate in vitro into mature adipocytes, allowing an insight into in vivo preadipocyte commitment.[202] Despite numerous studies conducted in C3H10T1/2 cell lines, no adipocyte specific commitment factor has been identified until now.

1.2.3.2.2 Preadipocyte growth arrest phase

In humans, adipocyte differentiation begins before birth, but most occurs shortly after birth; whereas in rodents, preadipocytes only begin differentiation after birth.[201, 203] Clonal cell lines are mostly used to study the pathways leading to the differentiation of committed preadipocytes. Primary preadipocyte cell cultures are not commonly used...
to study differentiation since these cells are morphologically similar to fibroblasts and are at different stages of differentiation. Besides, these cells also have a limited life span in culture and only constitute a small percentage of the total adipose tissue.\textsuperscript{201} Therefore, preadipocyte cell lines such as 3T3-L1 cells, already committed to the adipocyte cell lineage, are used instead. These cell lines have a homogenous cell population at the same differentiation stage, allowing uniform response to treatments.\textsuperscript{204}

3T3-L1 is a unipotent preadipocyte cell line which has undergone the determination phase and can either remain as a committed preadipocyte population, or undergo differentiation with hormonal induction.\textsuperscript{204} Differentiated 3T3-L1 cells can form fat tissue that is very similar to adipose tissue \textit{in vitro}, as well as \textit{in vivo} when implanted in athymic mice.\textsuperscript{205} Maximal differentiation is achieved by early hormonal induction of 3T3-L1 cells, by a defined adipogenic cocktail made of insulin, dexamethasone (DXM) and methylisobutylxanthine (MIX), and fetal bovine serum (FBS), collectively termed as MDI (MIX, DXM and Insulin).\textsuperscript{200, 201}

Within an hour of MDI induction \textit{in vitro}, 3T3-L1 preadipocytes start expressing the transcriptional factors CCAAT/enhancer binding protein (C/EBP) $\beta$ and $\delta$.\textsuperscript{206} After 24 hours, preadipocytes undergo a post-confluent mitosis called mitotic clonal expansion (MCE) and by day 2, enter into an unusual growth arrest called G_D.\textsuperscript{207} This growth arrest phase is necessary to unwind DNA, allowing transcription factors to induce or silence genes related to adipogenesis and adipocyte phenotype.\textsuperscript{201, 206} After day 2, growth arrested cells begin to express differentiation markers, such as PPAR-$\gamma$ and C/EBP-$\alpha$. This triggers changes in cell morphology from a fibroblast like shape to a spherical shape, followed by the ability to accumulate lipid droplets by terminal differentiation by day 5-7 of MDI induction.\textsuperscript{200}
Figure 3: Schematic representation of adipocyte differentiation. Abbreviations used: BMP: Bone morphogenetic protein, C/EBP: CCAAT/enhancer binding protein, FGF: Fibroblast growth factor, PPAR: Peroxisome proliferator-activated receptor, TGF: Transforming growth factor.
1.2.3.2.3 Terminal differentiation phase

Terminal differentiation is driven by the action of a number of transcription factors leading to activation of adipogenic genes.

(i) CCAAT/enhancer binding protein (C/EBP)

C/EBP transcription factors are characterized by their ability to bind to the CCAAT motif in several gene promoters, and have 5 isoforms- $\alpha$, $\beta$, $\delta$, $\varepsilon$ and $\xi$ (CHOP-10). C/EBP$\beta$ and $\delta$ are early regulators of differentiation while C/EBP-$\alpha$ is a late adipogenic regulator with PPAR-$\gamma$.\(^{[208]}\) CHOP-10 acts as a dominant negative C/EBP function and C/EBP-$\varepsilon$ has no known adipogenic function.\(^{[209]}\)

C/EBP-$\beta$ and $\delta$

C/EBP-$\beta$ and $\delta$ are the first transcription factors to be expressed after adipogenic induction. Overexpression of either C/EBP-$\beta$ or $\delta$ in 3T3-L1 preadipocytes accelerates adipocyte differentiation.\(^{[200]}\) On the other hand, mice lacking either C/EBP-$\beta$ or C/EBP-$\delta$ display reduced lipid accumulation and UCP-1 expression in their BAT, whereas WAT seems to develop normally. Deletion of both C/EBP-$\beta$ and C/EBP-$\delta$ causes severe impairment in adipocyte development with 15% survival rate only. Alive mice have severe reduction in BAT mainly due to reduced lipid accumulation, and moderate impairment in WAT tissue mass, due to reduced cell number, without alterations in the cell size or morphology.\(^{[210]}\) During mitotic clonal expansion of preadipocytes, C/EBP-$\beta$ is phosphorylated and activated by glycogen synthase kinase-3 (GSK-3) and mitogen activated protein kinase (MAPK), thus acquiring its DNA binding
potential.\textsuperscript{200} C/EBP-\(\beta\) and \(\delta\) directly induce expression of C/EBP-\(\alpha\) and PPAR-\(\gamma\), which are key transcriptional regulators of adipogenesis.\textsuperscript{211}

**C/EBP-\(\alpha\)**

C/EBP-\(\alpha\) is a pleiotropic transcriptional activator of genes specific to adipocytes. Its expression is induced by C/EBP-\(\beta\) and \(\delta\) within 2 days of MDI expression along with PPAR-\(\gamma\) expression.\textsuperscript{200} Although initial activation of C/EBP-\(\alpha\) is by C/EBP-\(\beta\) and \(\delta\), C/EBP-\(\alpha\) is also induced by PPAR-\(\gamma\), and once expressed, it can auto-activate itself to maintain its own expression. It can also bind to its site on the PPAR-\(\gamma\) promoter and mutually induce each other, forming a stable regulatory loop.\textsuperscript{200} C/EBP-\(\alpha\) acts synergistically with PPAR-\(\gamma\) to activate transcription of adipogenic genes like FABP4, leptin, SCD-1 and GLUT4.\textsuperscript{200} However, C/EBP-\(\alpha\) alone cannot induce adipogenesis, as observed when ectopic C/EBP-\(\alpha\) expression in mouse embryonic fibroblasts lacking PPAR-\(\gamma\), failed to induce adipogenesis, whereas PPAR-\(\gamma\) alone could initiate adipogenesis in C/EBP-\(\alpha\) deficient cells.\textsuperscript{212} Insulin has also been found to reduce C/EBP-\(\alpha\) mRNA and protein levels since two of the three phosphorylation sites of C/EBP-\(\alpha\) (Thr 222 and Thr 226) are insulin sensitive sites phosphorylated by GSK3.\textsuperscript{213} C/EBP-\(\alpha\) overexpression in 3T3-L1 cells can stimulate adipogenesis even without MDI induction, whereas blocking C/EBP-\(\alpha\) expression with an antisense C/EBP-\(\alpha\) RNA inhibits adipocyte differentiation.\textsuperscript{214, 215} C/EBP-\(\alpha\) null mice had defective lipid accumulation and died from hypoglycemia within 8 hours, proving its importance in adipogenesis.\textsuperscript{216}
(ii) Peroxisome proliferator activated receptor-γ (PPAR-γ)

PPAR-γ belongs to the nuclear hormone receptor super family of ligand activated transcription factors, and has two isoforms: the constitutively expressed γ1 and the adipose tissue restricted γ2. PPAR-γ is the master regulator of preadipocyte differentiation and plays a central role in the maintenance of mature adipocyte function and insulin sensitivity. Generation of PPAR-γ (-/-) mice leads to complete absence of adipose tissue, whereas PPAR-γ (+/-) mice are resistant to diet-induced obesity. Adenoviral expression of a dominant negative PPAR-γ into mature differentiated 3T3-L1 adipocytes caused dedifferentiation. Selective deletion of PPAR-γ in mature adipocytes of mice led to reduced cell viability, causing apoptosis within a few days. Humans with dominant-negative mutations in a single allele of PPAR-γ were found to develop partial lipodystrophy and insulin resistance.

PPAR-γ is responsible for activating adipogenic genes like adipose protein-2 (aP2), lipoprotein lipase (LPL), acyl CoA synthase and phosphoenolpyruvate carboxykinase (PEPCK), by binding to the peroxisome proliferator response element in the promoters of these target genes. Activated PPAR-γ directly targets adiponectin, which promotes fatty acid oxidation and insulin sensitivity. PPAR-γ also controls genes involved in glucose homeostasis by increasing GLUT expression. PPAR-γ can also be anti-inflammatory causing reduced TNF-α production. Although the transcriptional activity of PPAR-γ can be controlled by exogenous natural ligands such as long chain polyunsaturated fatty acids and synthetic ligands such as thiazolidinediones (TZD), identification of an endogenous ligand responsible for the regulation of PPAR-γ activity during adipogenesis has not been determined yet. SREBP-1c expression has been found to be up-regulated during early adipogenesis, suggesting a role in the production of the endogenous PPAR-γ ligand.
(iii) Sterol regulatory element binding protein-1c (SREBP-1c)

SREBP-1c is a pro-adipogenic, basic helix-loop-helix leucine zipper transcription factor implicated in stimulating endogenous PPAR-γ ligand production and inducing PPAR-γ expression.\textsuperscript{[229]} It is activated post-prandially by insulin and targets lipogenic genes that encode for enzymes like FAS, HMG-CoA synthase, LDL-receptor and acetyl CoA carboxylase (ACC).\textsuperscript{[230]} Adipose determination and differentiation factor 1 (ADD1) in rat adipocytes are homologous to human SREBP-1c.\textsuperscript{[229]}

SREBP-1c remains membrane bound in the endoplasmic reticulum until activation by cholesterol, which causes translocation to Golgi bodies for proteolytic cleavage. SREBP-1c is then released into the cytosol and enters the nucleus to bind to target genes through the sterol response element for gene activation.\textsuperscript{[230]} In vitro, insulin has been found to increase SREBP-1c mRNA levels in isolated rat hepatocytes, while glucagon has the reverse effect.\textsuperscript{[231]} In vivo, fasting mice with low insulin and high glucagon levels have reduced SREBP-1c mRNA levels and less activity of SREBP-1c target genes.\textsuperscript{[232]} During adipogenesis, SREBP-1c mRNA levels were found to be increased within the first day of MDI induction of 3T3-L1 preadipocytes.

Overexpression of SREBP-1c in these cells increased PPAR-γ mRNA levels.\textsuperscript{[229]} However, SREBP-1c null mice had normal adipose tissue, suggesting compensatory action by SREBP-2, another isoform of SREBP family capable of stimulating endogenous PPAR-γ ligand production.\textsuperscript{[233]}
1.2.3.3 Adipocyte turnover

Adipocyte turnover is a dynamic process that occurs throughout one’s life span. Adipose tissue can respond to excess energy by expanding up to 60-70% of body weight by two mechanisms: hypertrophy and hyperplasia.\[^{234}\] Hypertrophy entails an increase in individual adipocyte size due to enhanced storage of TG within lipid droplets. Excessive adipocyte hypertrophy can cause adipocyte dysfunction.

Hyperplasia entails an increase in total adipocyte number. Stem cells present in the adipose tissue proliferate and differentiate into new adipocytes which provides an alternative to adipocyte hypertrophy for storage of excess lipids in circulation.\[^{235}\] Rodents have a higher adipocyte proliferation rate than humans, where it is estimated that young adult mice generate approximately 15% new adipocytes per month.\[^{235}\] In humans, only around 10% of adipocytes are renewed annually at all adult stages.\[^{236}\] However, total adipocyte number is believed to be determined during childhood and adolescence and does not change with later weight gain or loss. Therefore, life-long obesity occurs primarily through adipose hyperplasia, while hypertrophy contributes to adult onset-obesity or obesity worsening.\[^{237}\]

1.2.3.4 Adipose tissue physiology

1.2.3.4.1 Regulation of glucose and lipid homeostasis

Adipose tissue serves a protective role as an insulating buffer and structural support for internal organs. However, its key metabolic functions in the body are the storage of excess circulating fatty acids during surplus and mobilization of TG during deficit, and secretion of adipocytokines.\[^{152}\]
1.2.3.4.1.1 Fatty acid transport into adipocytes

Circulating dietary TG packaged in chylomicrons (from the gut) and VLDLs (from the liver) are too large to enter adipocytes and therefore need to be hydrolyzed by the action of LPL.[238] They are then transported into the adipocyte by the action of proteins like CD36, fatty acid transport protein (FATP) and aP2/FABP4.

(i) CD36

CD36 (or fatty acid translocase in mice) is an integral membrane protein expressed on macrophages, adipocytes, hepatocytes and myocytes. It is primarily known as a scavenger receptor which has the ability to recognize foreign substances and as pathogens, thus playing a major role in immune defense, as well as many endogenous molecules and particles including oxidized LDLs.[239] However, in adipocytes it is considered an important facilitator of fatty acid uptake since CD36 null mice have defective fatty acid uptake and severely reduced fatty acid oxidation.[240]

CD36 is also a marker for adipose differentiation since implantation of CD36 gene silenced preadipocytes in athymic mice reduced de novo fat pad formation.[241] Mice with CD36 deletion are lean and protected from high fat diet induced obesity.[242] Conversely, muscle-specific overexpression of CD36 in mice causes enhanced fatty acid oxidation, reduces plasma TG levels and increases plasma insulin levels, and in MKR mice (transgenic mice with murine creatine kinase muscle (Ckm) promoter directing expression of the human IGF-1R gene containing the K1003R mutation), it reverses insulin resistance and diabetes.[243, 244] C/EBP-α plays a key role in the regulation of CD36 and overexpression of C/EBP-α increases CD36 mRNA and protein levels.[245]
(ii) Adipocyte protein 2 (aP2)

Also known as fatty acid binding protein 4 (FABP4), aP2 is expressed in adipocytes and macrophages, is the most abundant protein found in mature adipocytes and has been extensively used as a marker for adipocyte differentiation. aP2 is a cytoplasmic protein that binds to long-chain fatty acids and facilitates its trafficking into the adipocyte.\[246\] Its secretion is strongly regulated by lipolysis-related fatty acids.\[246\] The exact biological function of FABP4 is not yet fully understood, but FABP4 null mice are protected from obesity-induced insulin resistance and diet-induced atherosclerosis.\[247, 248\] Double knockout of both FABP4 and FABP5 in mice leads to defective uptake of fatty acids and compensatory up-regulation of glucose metabolism, resulting in hyperlipidemia during fasting states and hepatic steatosis.\[249\]

aP2 has recently been identified as a lipid activated adipocytokine expressed in macrophages, suggesting a role in adipose inflammation.\[250\] Circulating aP2 plasma levels were found to be high during obesity and could independently predict inflammation and fibrosis in NAFLD patients.\[251, 252\]

(iii) Stearoyl-CoA desaturase -1 (SCD-1)

SCD-1 is a δ-9 fatty acid desaturase, located on the endoplasmic reticulum membrane, and responsible for the synthesis of monounsaturated fatty acids (MUFA) from saturated fatty acids by introducing a double bond between carbon 8 and 10. SCD-1 activity produces palmitoleic acid and oleic acid, which are key substrates in the synthesis of phospholipids and TGs.\[253\] SCD-1 expression is regulated by nutritional and hormonal signals. Insulin, glucose, cholesterol and fatty acids induce SCD-1
expression, while n-3 and n-6 polyunsaturated fatty acids inhibit SCD-1 expression. Insulin and PPAR-γ have been found to induce SCD-1 expression. SCD-1 deficient mice had low MUFA levels, leading to decreased TG and fatty acid synthesis. Loss of SCD-1 function increased insulin sensitivity and reduced hepatic steatosis and adipocyte inflammation, protecting the mice from diet-induced obesity. Overexpression of SCD-1 in myocytes induces abnormal lipid partitioning and progression to obesity in lean humans.

(iv) Lipoprotein Lipase (LPL)

LPL is synthesized in adipose, skeletal and cardiac parenchymal cells, but it functions by interacting with heparin sulphate proteoglycans (HSPG) at the endothelial luminal surface. LPL is responsible for hydrolyzing TG in chylomicrons and VLDLs into fatty acids, which are then transported into adipocytes. LPL is an important marker for adipogenesis. SREBP-1c and PPAR-γ initiate the transcription of LPL gene, whereas Angiopoietin-like-4 catalyzes LPL conversion into inactive monomers. TNF-α inhibits LPL transcription by blocking the interaction of the nuclear factor Y with the CCAAT box on the LPL gene promoter in the nucleus. LPL is also nutritionally regulated, with high activity observed in postprandial conditions in association with high circulating insulin levels, and reduced activity during fasting state when insulin levels are low.

LPL null mice have high plasma TG and VLDL levels at birth, and die due to hypoglycemia stemmed from their inability to hydrolyze dietary lipids. LPL sensitivity to insulin and feeding is reduced during obesity, despite an increase in LPL mRNA expression per adipocyte.
(v) Angiopoietin-like 4 (Angptl4)

Angptl4 is mostly expressed in adipose tissue in mice, but in humans it is ubiquitously expressed. Angptl4 plays an important role in the clearance of circulating lipids by regulating the activity of LPL. Angptl4 inactivates LPL by binding and converting it from an active dimer to an inactive monomer, thus hindering LPL-HSPG interaction at the cell surface and increasing TG levels in the plasma.

Overexpression of Angptl4 in transgenic mice led to significant hypertriglyceridemia and reduced LPL activity, whereas knockdown of Angptl4 caused reduced plasma TG levels and enhanced LPL activity. Angplt4 expression is stimulated by fasting or NEFA via the activation of PPAR-γ. Although a direct effect of Angptl4 on inflammation pathways has yet to be identified, it has recently been associated with regulation of inflammation since it decreases the expression of pro-inflammatory adipokine IL-6 and serum amyloid A, as observed in Angptl4 knockout mice which displayed increased serum levels of these pro-inflammatory adipokines and hepatic macrophage infiltration.

1.2.3.4.1.2 Triacylglycerol (TG) formation in adipocytes

Fatty acids are stored in lipid droplets of adipocytes as TG, which is constituted by three fatty acid side chains esterified to a glycerol backbone. Since adipocytes lack glycerol kinase, TG formation in adipocytes requires glucose in order to produce glycerol-3-phosphate and dihydroxyacetone phosphate (DHAP) produced during glycolysis.

Fatty acids trafficked into the adipocytes are activated into acyl-CoA by acyl-CoA synthetase and one acyl-CoA is esterified onto glycerol-3-phosphate by the action of
glycerol-3-phosphate acyltransferase (GPAT) in the mitochondria and endoplasmic reticulum to produce lysophosphatidic acid. The esterification of the second acyl-CoA is catalyzed by 1-acylglycerol-3-phosphate acyltransferase (AGPAT) in the endoplasmic reticulum to produce phosphatidic acid. Phosphatidic acid can then either be used to synthesize other phospholipids or to produce TG. Phosphatidic acid phosphatase (PAP) catalyzes the conversion of phosphatidic acid into intermediate 1, 2-diacylglycerol form. The last acyl-CoA is esterified by the action of diacylglycerol acyltransferase (DGAT) to produce TG. Overexpression of DGAT2, an isoform of DGAT in 3T3-L1 adipocytes led to an increase in MUFA accumulation, TG content and enhanced expression of genes related to fatty acid synthesis like SCD-1.

1.2.3.4.1.3 Lipid droplet formation in adipocytes

TG produced in the endoplasmic reticulum enters the cytoplasm by budding off as lipid droplets composed of a large TG core surrounded by an osmophilic phospholipid layer. The main purpose of these lipid droplets is the regulation of lipid homeostasis by safely storing fatty acids when excessive, and releasing them into circulation when energy is required.

Perilipin (plin) proteins, whose expression is regulated by PPAR-γ, coat the lipid droplets and regulate lipid droplet turnover by either protecting them from lipolysis or recruiting HSL to the lipid droplet surface to induce lipolysis.
(i) Adipose differentiation-related protein (ADRP)

ADRP, also known as adipophillin, is a 50kDa lipid droplet associated protein found in most cells and tissues, but is expressed rapidly at high levels in adipose tissue during adipocyte differentiation. ADRP belongs to the PAT family (named after its founding members- perilipin, ADRP and TIP47), which are lipid-droplet coating proteins that associate primarily with lipid droplet surfaces. ADRP is found to be up-regulated at sites of increased lipid accumulation such as fatty liver.

During adipogenesis, ADRP is highly expressed in 3T3-L1 preadipocytes from early stages of differentiation, due to up-regulation by PPAR-γ. However, towards the later stages of adipogenesis, despite no changes in ADRP gene expression, ADRP protein levels decrease and perilipin protein levels increase, suggesting that with increased TG accumulation in the adipocytes, perilipin takes over as the primary lipid droplet associated protein.

High-fat fed mice treated with antisense ADRP oligonucleotide displayed decrease in TG levels, but increased insulin action, thereby protecting the mice from diet-induced insulin resistance. However, ADRP-deficient mice show no difference in fat pad mass and body weight. On the other hand, adenoviral overexpression of ADRP in murine fibroblasts induces lipid accumulation with no change in expression of adipogenic genes, suggesting ADRP does not affect adipogenesis.
1.2.3.4.1.4 Lipolysis

During periods of energy deficit like fasting or exercise, TG stored in the lipid droplets inside adipocytes are rapidly hydrolyzed via lipase action to produce free fatty acids, which are released into the circulation to meet the energy demands of vital organs. In adipocytes, TG hydrolysis occurs by the action of three lipase enzymes: adipocyte triacylglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MGL).

(i) Adipose Triglyceride Lipase (ATGL)

Adipose triglyceride lipase (ATGL), also known as desnutrin or patatin-like phospholipase domain-containing protein 2 (PNPLA2), is one of the key enzymes involved in TG hydrolysis and is highly expressed in adipose tissue, and moderately expressed in skeletal and cardiac muscles.[285] It is the first and rate-limiting enzyme that selectively catalyzes the sequential hydrolysis of TG in adipocytes, producing diacylglycerols (DAG) and free fatty acids, by cleaving the first ester bond.[286]

ATGL expression is increased during adipose differentiation and its activity is tightly regulated by nutritional and hormonal signals since it is down-regulated by food intake, insulin and TNF-α, and is induced by fasting, glucocorticoids and PPAR-γ agonists.[287-289] Activator protein CGI-58, also known as α/β hydrolase domain-containing protein 5 (ABHD5), has been found to be the sole lipase activator for ATGL functioning via cAMP-dependent PKA pathway, whereas G0S2 has been found to inhibit ATGL activity in adipocytes.[285,290]
Overexpression of ATGL increases lipolysis with increase in glycerol and non-esterified fatty acid (NEFA) release, while antibody mediated ATGL inactivation or ATGL knockout mice inhibit TG hydrolase activity, and reduces lipolysis with decreased glycerol release. ATGL deficient mice have increased fat pad mass and body weight, since the adipose tissues are incapable of mobilizing sufficient amounts of fatty acids. Lack of ATGL also prevents hepatic endoplasmic reticulum stress, thus protecting from NAFLD, while ATGL overexpression increases fatty acid oxidation and induces hepatic steatosis.

(ii) \textit{G}_0/\textit{G}_1 \textit{Switch gene 2 (G0S2)}

Initially named due to its association with re-entry of cells from \textit{G}_0 to \textit{G}_1 phase in the cell cycle of mononuclear cells, G0S2 mRNA levels were found to be highly expressed in adipose tissue, particularly during adipogenesis. It is a dose-dependent dominant inhibitor of ATGL in adipocytes. G0S2 expression is increased by insulin and down-regulated by chronic treatment with TNF-\(\alpha\). G0S2 expression is reduced in obese individuals with poorly controlled T2D. Knockdown of the G0S2 gene inhibited adipogenesis and accelerated gluconeogenesis. G0S2 knockout mice have normal appearance, but have less adipose mass, impaired adipogenesis and enhanced adipose lipolysis and are resistant to diet induced liver steatosis. Overexpression of G0S2 accelerated adipogenesis, inhibits fatty acid oxidation and causes liver steatosis.
(iii) Hormone Sensitive Lipase (HSL)

Hormone sensitive lipase (HSL), an 84 kDa protein, is an intracellular neutral lipase with broad substrate specificity, capable of hydrolyzing TG, DAG and cholesteryl esters. HSL hydrolyzes DAG ten times faster than TG, therefore HSL deficiency results in DAG accumulation in adipose tissue. HSL is found freely in the cytosol, but is expressed the highest in the adipose tissue, and to a lesser extent in adrenal, ovary and testes.

HSL activation is regulated by hormones like catecholamines and insulin. During fasting, catecholamine levels increase and activate G-coupled receptors, which activate adenylyl cyclase to generate cAMP. Increased cAMP-dependent PKA pathway phosphorylates HSL and translocates it to the lipid droplets. Perilipin, a protein coating the lipid droplets responsible for the regulation of lipid droplet turnover, recruits HSL to the lipid droplet surface to trigger the lipolytic activity of HSL. No differences in body weight are observed in HSL-null mice, despite decreased adiposity and increased DAG accumulation in the adipose tissue, muscles and testis.

1.2.3.4.1.5 De novo lipogenesis in adipocytes

De novo lipogenesis in adipocytes does not contribute much to the overall TG synthesis in the adipocytes. However, unlike de novo lipogenesis in liver, which causes steatohepatitis, in the adipose tissue it has been found to be beneficial by improving insulin sensitivity and glucose homeostasis. In mice with genetically silenced aP2, an increase in de novo lipogenesis was observed, making the mice resistant to diet-induced obesity, NAFLD, insulin resistance and T2D by stimulating muscle insulin action and suppressing hepatosteatosis.
1.2.3.4.2 Adipose tissue as an endocrine gland and source of cytokines and chemokines

In addition to maintaining energy homeostasis in the body, white adipose tissue is also considered an active endocrine gland since it secretes a variety of hormones called adipokines, which have important effects on cellular metabolism. These include leptin, adiponectin, resistin, tumor necrosis factor-α (TNF-α), visfatin, interleukin-6 (IL-6), plasminogen activator inhibitor 1 (PAI1), monocyte chemoattractant protein-1 (MCP-1), angiotensinogen, retinol-binding protein-4, serum amyloid A, vaspin and FGF21. Adiponectin and leptin are produced primarily by adipocytes and have structural similarity to cytokines such as TNF-α, IL-6 and MCP-1, and are therefore termed adipocytokines.[303] Most adipocytokines have pro-inflammatory functions, except adiponectin, which is anti-inflammatory.

(i) Adiponectin

Adiponectin, also known as complement-related protein 30 (ACRP30), adipoQ, adipose most abundant gene transcript (apM1) and GBP-28, is one of the major adipocytokines. Adiponectin is highly expressed during adipocyte differentiation and is found in high levels in circulation in healthy individuals.[304] Its production and serum levels have been found to be inversely correlated to obesity, with decreased plasma levels observed with obesity, T2D and NAFLD.[305]

Adiponectin has been shown to ameliorate whole body insulin sensitivity via enhancement in fatty acid oxidation and glucose uptake in myocytes and suppression of hepatic gluconeogenesis through activation of the AMPK signaling.[246, 306] Adiponectin also has anti-inflammatory functions, where it suppresses the production of TNF-α in
obese mice by inhibiting the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and stimulating the production of anti-inflammatory IL-10 instead.\textsuperscript{246, 307}

Exogenous administration of adiponectin has been found to partially reverse insulin resistance in obese mice, while overexpression in transgenic mice leads to insulin sensitivity and alleviation of NAFLD.\textsuperscript{308, 309} Adiponectin deficient mice also develop hepatic insulin resistance and inflammation due to increased TNF-\(\alpha\) levels as well as reduced sensitivity to PPAR-\(\gamma\).\textsuperscript{310} TNF-\(\alpha\) and IL-6 suppress adiponectin expression in adipocytes, whereas PPAR-\(\gamma\) ligands stimulate adiponectin expression.\textsuperscript{225, 311}

\begin{enumerate}[label=(\roman*)]
\item Leptin
\end{enumerate}

Leptin (16 kDa) is a potent adipocytokine encoded by the obese (ob) gene. It is mainly expressed by adipocytes, but it is also found in mammary glands, gastric wall, placenta, ovaries, skeletal muscles, liver and pituitary gland. Leptin has many functions including regulation of appetite and energy balance, insulin sensitivity, activation of inflammation and reproduction and growth control.\textsuperscript{312, 313} The effects of leptin on food intake and energy expenditure are mediated by signaling in the hypothalamic region of the central nervous system.\textsuperscript{314}

Leptin also has pro-inflammatory functions due to its structural similarity to cytokines like IL-6.\textsuperscript{315} Leptin stimulates the production of IL-6, TNF-\(\alpha\) and MCP-1 from immune cells.\textsuperscript{316} Conversely TNF-\(\alpha\) can induce the expression of leptin and its receptors.\textsuperscript{317} Up-regulation of leptin expression in WAT as well as elevated levels of leptin in circulation is commonly observed during obesity and inflammation. Despite increased leptin levels, excessive adiposity is not reduced, suggesting leptin
Animal models for leptin deficiency have played a key role in the determination of the functions of leptin. Murine studies in the leptin deficient (ob/ob) mice and leptin receptor deficient (db/db) mice found that loss of leptin function caused hyperphagia, obesity and insulin resistance in these mice models. In normal rats with no leptin deficiency, exogenous leptin administration enhanced fatty acid oxidation in myocytes and hepatocytes and lowered hepatic lipogenesis, suggesting that increased leptin expression could cause lower hepatic steatosis.

Multiple spliced isoforms of the leptin receptor allows leptin to mediate its different functions by activation of the PI3K/AKT pathway to induce insulin sensitivity, and JAK/STAT3 pathway to mediate the pro-inflammatory responses of leptin.

(iii) Tumor Necrosis Factor-α (TNF-α)

Although primarily expressed in monocytes and macrophages, pro-inflammatory TNF-α is highly expressed in adipose tissue of obese mice and humans and is strongly associated with peripheral insulin resistance. TNF-α expression in adipocytes is stimulated by fatty acids, inducing lipolysis and hepatic lipogenesis, by activation of extracellular signal kinase. Exogenous TNF-α administration inhibits the insulin signaling pathway either by serine phosphorylation of insulin receptor substrate-1 (IRS-1) via the JNK1 pathway, or degradation of IRS-1 via the suppressor of cytokine signaling-3 (SOCS-3).

Further studies using genetic mouse models of TNF-α deficiency confirmed the negative effects of TNF-α on insulin signaling, since TNF-α (-/-) mice were protected from obesity-induced insulin resistance. In vitro studies also demonstrated that TNF-α treatment of 3T3-L1 adipocytes down-regulated the expression of adipocyte specific
genes such as HSL, adiponectin, C/EBP-α, PPAR-γ, and GLUT4, and up-regulated leptin expression via activation of nuclear factor NFKB.\textsuperscript{[226, 327]}

(iv) Interleukin-6 (IL-6)

IL-6 is a pro-inflammatory adipocytokine that is more expressed in visceral adipocytes compared to subcutaneous adipocytes.\textsuperscript{[328]} IL-6 expression in adipose tissue and circulatory levels of IL-6 are found to be increased during obesity and T2D and have been associated with insulin resistance.\textsuperscript{[329]} Polymorphism of the IL-6 gene in humans, leading to reduction in plasma IL-6 levels, was associated with greater insulin sensitivity after a glucose load.\textsuperscript{[330]} In addition, it has been shown from rodent studies that exogenous administration of IL-6 in mice blocks hepatic insulin signaling through inhibition of IRS-1 phosphorylation via SOCS-3 up-regulation, leading to hepatic insulin resistance.\textsuperscript{[331]}

However, IL-6 deficient mice develop obesity, high leptin levels, hepatic inflammation and insulin resistance, suggesting that similarly to leptin, IL-6 can also influence energy expenditure and feeding through the central nervous system.\textsuperscript{[332]} A C-174G promoter polymorphism in the IL-6 gene in healthy Finnish subjects was found to lower energy expenditure and insulin sensitivity.\textsuperscript{[333]} In pregnant mice, IL-6 deficiency caused reduced expression of energy balance regulating anabolic and oxerigenic peptides, suggesting a role for IL-6 in modulating the hypothalamic expression of these neuropeptides.\textsuperscript{[334, 335]}

IL-6 expression is stimulated by leptin, and increased IL-6 expression in adipocytes can suppress the expression of adiponectin.\textsuperscript{[225, 316]} IL-6 has a role in the recruitment and differentiation of T-cells, and IL-6 null mice have impaired immune and acute phase
Interestingly, immediately after exercise, IL-6 released from skeletal muscles is augmented. This fact has been associated with enhanced insulin action, via AMPK activation, promoting glucose uptake and fatty acid oxidation.\cite{337}

(v) Monocyte chemoattractant protein-1 (MCP-1)

Also known as chemokine (C-C motif) ligand 2 (CCL2), it is a monomeric chemokine peptide belonging to the C-C chemokine family, and belongs to a family of four MCP proteins (MCP-1, 2, 3 and 4). MCPs are produced by various cell types including endothelial, fibroblast, epithelial, smooth muscle, mesangial, astrocytic, monocyctic and microglial cells, either constitutively or after selective induction by oxidative stress, cytokines or growth factors, but are mainly secreted by macrophages.\cite{338} MCP-1 carries the CCL2 receptor CCR2 (CC chemokine receptor 2) on their surfaces and are therefore the main chemokine responsible for attracting monocytes into foci of active inflammation.\cite{339}

An increase of MCP-1 as well as macrophage infiltration was observed in both white adipose tissue and plasma of obese mice, suggesting that adipose tissue in obesity could be characterized by chronic low-grade inflammation.\cite{340} MCP-1 secretion from adipocytes was found to directly trigger macrophage infiltration into adipose tissue, resulting in inflammation, systemic insulin resistance and increase hepatic triacylglycerol content.\cite{341}

Knockout of MCP-1 gene, inhibition of MCP-1 expression by a dominant-negative mutant or knocking out MCP-1 receptor CCR2, in diet-induced and genetically obese mice attenuated macrophage infiltration and inflammation in adipose tissue, and increased insulin sensitivity and hepatic steatosis.\cite{341, 342} Increased MCP-1 expression
in adipose tissue precedes the expression of other macrophage markers in obesity, and an increase in circulating MCP-1 is sufficient to induce insulin resistance.\textsuperscript{[343, 344]} MCP-1 expression is higher in visceral adipocytes, compared to subcutaneous adipocytes.\textsuperscript{[345]} MCP-1 is an insulin responsive gene that decreases insulin stimulated glucose uptake, down-regulates LPL expression and increases the expression of adipogenic genes.\textsuperscript{[340, 344]} MCP-1-induced protein (MCPIP) also promotes adipogenesis with an increase in endoplasmic reticulum stress, ROS production and inducible nitric oxide synthase (iNOS) expression.\textsuperscript{[346]}

1.2.3.4 Pathophysiology of adipose tissue dysfunction and inflammation

Pathological obesity is associated with adipose tissue expansion and dysfunction, accompanied by chronic low grade inflammation.\textsuperscript{[1]} Physiologically, adipose tissue responds to short-term excess nutrients by hyperplasia and hypertrophy of subcutaneous adipocytes, which safely stores the excess fatty acids as TG in lipid droplets.\textsuperscript{[234]} However, environmental and genetic factors can lead to chronic over-nutrition and reduced energy expenditure, causing persistent increase in nutrient supply.\textsuperscript{[1]} This nutrient-rich environment first causes subcutaneous adipocyte expansion and then abnormal lipid partitioning to visceral adipocytes when subcutaneous adipocytes reach their limits (Figure 4).\textsuperscript{[347]}

Over-expansion of adipocytes has been associated with activation of nuclear factor-kappa light chain enhancer of activated B-cells (NF-κB) and c-Jun n-terminal kinases (JNK) signaling pathways. Other factors such as ceramide, elevated fatty acids which bind and activate toll like receptor 4 (TLR4), receptor for advanced glycation end-products (RAGE) and cellular stresses such as reactive oxygen species (ROS) and ER
stress have also been found to activate JNK and NF-κB pathways. JNK activation promotes phosphorylation of IRS-1, which negatively regulates insulin receptors, while activation of NF-κB leads to its translocation to the nucleus, triggering expression of various mediators and markers of inflammation, resulting in insulin resistance. \[348\]

Obesity-induced insulin resistance has also been attributed to various other factors including adipose tissue hypoxia, ectopic fat deposition in organs such as the liver and muscles, angiogenesis, mitochondrial dysfunction and increased inflammation.\[349-356\]

In the adipocytes, increased production of pro-inflammatory adipokines leads to the suppression of adipogenic genes like adiponectin, ATGL, C/EBP-α, PPAR-γ and HSL, and recruitment of immune cells like macrophages to adipose tissue, creating a milieu of chronic low grade inflammation and insulin resistance.\[235, 311, 323, 339\]

Visceral adipocytes also exacerbate inflammation since they are less responsive to insulin and secretes more MCP-1 and IL-6 than subcutaneous adipocytes.\[328, 345\] Free fatty acids and adipokines like TNF-α and IL-6 play a prominent role in the development of insulin resistance by blocking the insulin signaling pathway and attenuating adipose differentiation and lipid uptake.\[325, 336\] Increased visceral adipose tissue is associated with increased insulin resistance.\[349\] This is due to the close proximity of visceral adipocytes to the liver, since free fatty acids released by these adipocytes during lipolysis and dysfunction enter the liver, causing insulin resistance, increased TG accumulation and hyperinsulinemia.\[347\]

Adipose dysfunction can also lead to ectopic lipid deposition into non-adipose tissues like liver, pancreas and heart, which might lead to steatosis and organ dysfunction and eventually insulin resistance, T2D, NAFLD and cardiovascular diseases.\[347\]
Figure 4: Pathogenesis of adipose tissue dysfunction during obesity. Abbreviations used: CD: Cluster of differentiation; FFA: free fatty acids; IL: Interleukin; MCP-1: monocyte chemoattractant protein-1; TNF-α: Tumor necrosis factor-alpha.
1.3 Animal model: *Alms 1* mutant (*foz/foz*) mouse model

The *fat aussie* (*foz*) mouse has been identified to have a spontaneous mutation (*foz*) of an 11 bp truncating deletion in exon 8 of the *Alms 1* gene.[35] Mutations in the ALMS1 gene in humans are responsible for the development of Alström syndrome, which is a rare autosomal recessive condition affecting multiple organs. Individuals carrying an ALMS1 mutation develop progressive loss of vision and hearing, obesity-related T2D and hypertriglyceridemia. Some individuals also develop hypothyroidism, infertility, low growth hormone levels, kidney failure, dilated cardiomyopathy, congestive heart failure and fatty liver disease.[357, 358]

The *fat aussie* (*foz/foz*) mice develop a similar phenotype to the human syndrome. The *foz/foz* mice are hyperphagic and underactive and develop obesity by 100-120 days of age on a standard chow diet.[35] Both genders of mice develop T2D, however, female mice have a later onset of the disease. Significant hyperinsulinemia precedes the development of hyperglycemia in these mice in response to insulin resistance. High fat (HF) feeding does not change serum TG levels; however, cholesterol levels rise up to 200% in the *foz/foz* mice compared to wild-type mice.[35] The *foz/foz* mice also develop hepatomegaly with moderate to severe steatosis. Male *foz/foz* mice are infertile, while females develop infertility after onset of obesity. Therefore, heterozygous mice are normally used for breeding purposes.[35]

Further characterization of these mice revealed that female *foz/foz* mice fed on a high-fat diet until 180 days of age develop NASH. This is characterized by hepatomegaly, steatosis, increased ALT levels, hepatic inflammation, fibrosis and severe hepatocellular ballooning degeneration, whereas wild-type littermates only developed simple
steatosis. Therefore, the \textit{foz/foz} mice serves as a mouse model of fatty liver disease, in which high-fat feeding triggers NAFLD progression to NASH.

The metabolic phenotype of the \textit{foz/foz} mice varies depending on genetic background. Female NOD.B10 mouse with the \textit{foz/foz} mutation fed on high-fat diet for 24 weeks exhibit attenuation in subcutaneous adipose tissue expansion, termed as adipose restriction. This is associated with WAT inflammation, hypoadiponectinemia, moderate hyperglycemia and NAFLD progression to NASH. Conversely, female Balb/c mice with the \textit{foz/foz} mutation fed on the same diet, develop identical excessive weight gain as the female NOD.B10 \textit{foz/foz} mice, but their adipose depots expand proportional to body weight. They remain normoglycemic and display no decrease in adiponectin levels or development of NASH (unpublished data). These dramatic strain differences present a great opportunity to study the pathophysiological basis underlying the abnormal metabolic responses to over-nutrition in the development of T2D and NASH.
1.4 Rationale

In response to chronic overfeeding, some individuals develop healthy obesity; whereas others develop obesity in association with metabolic syndrome and have an increased risk for the development of conditions such as T2D and NASH. Adipose tissue inflammation and dysfunction is often associated with both these conditions. Unknown is the role of adipose tissue in determining whether the result of overfeeding is healthy or unhealthy obesity. For example, it is not known if a primary defect in adipose tissue in response to overfeeding results in early dysfunction of this tissue (e.g. adipose restriction and inflammation) with the secondary consequences of insulin resistance, islet β-cell failure, T2D and/or NASH.

Alternatively, the adipose tissue dysfunction could be secondary to, for example, primary failure of islet β-cells to chronic overfeeding, hyperglycemia and then hyperglycemia-induced toxicity of adipose tissue. The Alms 1 mutant mouse on the Balb/c and NOD.B10 backgrounds provide an excellent model for studying the role of adipose tissue in overfeeding induced obesity, metabolic syndrome and T2D and NASH development. While both strains of mice overfeed and develop obesity, in response to the additional insulin of HF feeding, only NOD.B10 mutant mice develop T2D and NASH. Balb/c mutant mice become obese but do not develop these conditions suggesting the obesity of Balb/c mice is healthy obesity.
1.5 Hypothesis and Aims

This thesis is part of a bigger project with the overall aim to characterize over time, the early adipose tissue responses (expansion and restriction), in parallel to the development of insulin resistance, islet β-cell responses to the insulin resistance (compensation and decompensation), hepatic steatosis and NASH, as it develops in high-fat fed NOD.B10 foz/foz and Balb/c foz/foz female mice. The work is being performed in order to identify the relationship between β-cell decompensation, adipose tissue dysfunction and the onset of T2D and NASH.

Within this broader aim, this longitudinal study focuses on the role of adipose tissue in the development of T2D and NASH.

1.5.1 Hypothesis:

A primary defective response of adipose tissue to chronic overfeeding in NOD.B10 mice underlies its propensity to develop T2D and NASH; whereas a healthy adipose tissue response to overfeeding in Balb/c mice underlies its resistance to develop these metabolic conditions.
1.5.2 Aims:

The overall aim is to determine the role of adipose tissue in the determination of a healthy obesity response or obesity associated with metabolic diseases in Balb/c mice compared to NOD.B10 mice.

Specific Aims:

1. To determine the early response and time-course of adipose tissue expansion, gene expression and inflammation, together with a secreted adipokine profile, of NOD.B10 and Balb/c mice, to the overfeeding challenges of HF diet, the flox/flox genotype and the combination of both.

2. To determine the temporal relationship between the adipose tissue responses to the overfeeding challenges in the two mice strains and the development of T2D and NASH.
2. Materials and Methods

2.1 Breeding history

The *fat aussie* (*foz*) mouse model was first identified in a transgenic mice strain expressing the Fas ligand (FasL) in pancreatic beta cells under control of the human insulin promoter (HIP) in the Non-obese diabetic (NOD) mice background, known as the HIP-FasL NOD transgenic mice. These mice displayed obesity and hyperglycaemia by age of 100-120 days, which was associated with a spontaneous *Alms1* mutation. Homozygous mutant mice developed sterility upon onset of obesity, hindering breeding. Therefore, for mapping purposes, the *foz* mutation was outcrossed to a C57Bl/10 mouse strain. Since this phenotype segregated as a Mendelian monogenic recessive trait, a two generation outcross-intercross breeding strategy was applied. The mutation was outcrossed from the NOD mice to a B10.Br background and F1 progenies were intercrossed to breed the mutation in F2 progeny. These NOD.B10 (mixed NOD X B10.Br backgrounds) mice revealed the 11bp deletion on exon 8 and were homozygous for the *foz* mutation. The *fat aussie* phenotype observed in the NOD.B10 mice were recapitulated into the Balb/c mice strain. NOD.B10 heterozygous *foz/+* mice were bred with Balb/c +/- wild type mice to obtain an F1 generation of 50% Balb/c heterozygous *foz/+* mice. These mice were then back-crossed with the Balb/c wild type +/- mice over 9 generations to get >99.5% Balb/c heterozygous *foz/+* mice.

2.2 Housing, diet and ethics

Mice were maintained under specific pathogen-free conditions in an artificial 12-hour light/dark cycle, and were accommodated at a density of 2-5 mice per cage in a
temperature controlled room of 21-24°C with 40-60% humidity. Mice were fed on a
normal chow diet (59.8% carbohydrate, 20.0% protein and 5.4% fat by weight; energy
content 12MJ/g; Gordon’s Specialty Stockfeed, NSW, Australia) with free access to
autoclaved tap water.

For this study, heterozygous male and female mice from NOD.B10 and Balb/c strains
were time-mated during 3 consecutive nights at the Animal Facility of The Canberra
Hospital and Animal Facility at the Hugh Ennor building at the Australian National
University, to produce foz/+ (heterozygous), foz/foz (foz mice) and +/- (wild type) mice.
Pups were born after 21 days of gestational period. At 3 weeks of age, mice were
weaned on a normal chow diet with free access to autoclaved tap water.

All animal procedures were approved by the Animal Experimental Ethics Committee at
the Australian National University (Project A2011/27) and all animals received care in
compliance with the guidelines prescribed by this committee. The focus of this study
was directed towards the female mice since they have a slower progression towards
T2D and NASH than the male mice, allowing better characterization of the
pathophysiology of the diseases. The pups were identified using ear notch perforations
(see ear notch chart in Appendix section). The ear biopsies were obtained under aseptic
conditions and used to genotype the mice for the foz mutation.

2.3 Genotyping

2.3.1 DNA extraction

DNA samples from ear notches were obtained at 3 weeks of age. Each ear tissue was
individually lysed overnight or over 4 hours by incubation at 65°C on a dry bath
incubator (MD-02-220, Major Science, Taiwan) in a solution containing 400 µL of
Lysis buffer (see Appendix for solution preparation) mixed with 4 µL of Proteinase K (Serine protease, Cat # 03115828001, Roche, Mannheim, Germany). Samples were then vortexed (Ratex Vortex mixer, VIC, Australia) to break any tissue clumps and centrifuged at 14,800 rpm for 10 min at 4°C (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA). The supernatant was then transferred to a new set of microtubes containing 400 µL ice-cold isopropanol (Cat# 425, Univar, Ajax FineChem, NSW, Australia). DNA was precipitated after centrifugation at 14,800 rpm for 10 min at 4°C (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA). The supernatant was then removed and the DNA pellet washed with 1 mL of cold 70% ethanol (see Appendix), followed by centrifugation at 14,800 rpm for 10 min at 4°C (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA). Supernatant was discarded and the DNA pellet was left to dry inside a fume hood (Dyna Safe control system MK3, Dyna Flow, NSW, Australia) for 20 min to evaporate any remaining ethanol. The DNA pellet was resuspended in 300 µL of Tris-EDTA (TE) buffer (see Appendix) and incubated on a 65°C dry bath incubator (Cat# MD-02W-220, Major Science, Taiwan) for 10 min. The pellet was homogenized by vortexing (Ratex Vortex mixer, VIC, Australia) and stored for further analysis.

2.3.2 Polymerase Chain Reaction (PCR) analysis

The resultant DNA extracted from ear notches were used in a PCR reaction for identification of the 11 bp deletion of exon 8 on the Alms1 gene as previously described.[378] Primers flanking the 11 bp deletion of exon 8 on the Alms1 gene were purchased from GeneWorks (GeneWorks Pty. Ltd, SA, Australia). The primer sequence used was forward primer: 5’ ACA ACT TTT CAT GGC TCC AGT 3’ and reverse primer: 5’ TTG GCT CAG AGA CAG TTG AAA 3’.
Briefly, 1 µL of DNA sample was mixed in 12.5 µL Biomix™ Red (using *Taq* DNA Polymerase, cat# BIO-25006, BioLine, NSW, Australia), 0.5 µL of forward primer, 0.5 µL of reverse primer and 10.5 µL of Milli Q water. DNA was amplified using a PCR machine (iCycler, BioRad, CA, USA) under the following conditions: initial denaturation at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min for denaturation, annealing and extension, respectively.

The resulting PCR products were separated on a 4% agarose gel by electrophoresis. 4 g of agarose (Cat# 16103102, BioRad, CA, USA) was added to 100 mL of 1X TAE buffer (Tris-Acetic acid-ethylenediaminetetracetic acid buffer) (see Appendix) and dissolved in a microwave by heating for 2 min. The gel was cooled down to around 60°C prior to addition of 10 µL ethidium bromide at 10 mg/mL (Cat# 161-0433, BioRad, CA, USA), and set onto the gel chamber with a set of combs. After solidification, the combs were removed and the gel was assembled into the electrophoresis tank (BioRad wide mini sub cell GT, BioRad, CA, USA). 1X TAE buffer was then added to the gel chamber. 5 µL of ethidium bromide (Cat# 161-0433, BioRad, CA, USA) was added to the bottom chamber of the tank to improve visibility of the DNA bands. 10µL of PCR product was added to each well and electrophoresis was performed at 180V for 20min using the Power Pac Universal power supply (500V/2.5A/500W, BioRad, CA, USA). DNA bands were visualized under UV light (Gel Doc XR System PC, BioRad, CA, USA) and analyzed by Vision Capture 14.2 software. Visualization of DNA bands allowed classification of the mice into heterozygous (HET), homozygous wild type (WT) and homozygous *foz/foz* (*foz*) mice according to the size and number of the DNA fragments amplified by the PCR.
2.4 Experimental time line and diet composition

At 4 weeks of age, female wild-type and foz/foz (*Alms1* mutant) littermates from both NOD.B10 and Balb/c strains were randomly distributed into two diet groups- standard chow (5.4% fat), or high-fat diet (41% carbohydrate, 22.6% protein, 23.5% fat by wt; energy content 20MJ/kg; SF03-020, Specialty Feeds, Glen Forrest, Australia)(Figure 5). NOD.B10 mice groups were assigned to tissue harvesting at increasing ages of 6, 8, 10 and 12 weeks, whereas Balb/c mice at 6 and 12 weeks of age only (Figure 6). Balb/c mice were considered as controls, resistant to T2D and/or NASH and therefore no major changes were expected to occur between the two time-points (Figure 7).

2.5 Body weight and glucose monitoring

Body weight and non-fasting blood glucose levels of conscious mice were monitored every fortnight from 4 to 12 weeks of age (9-10 AM). Body weight was recorded on a weighing scale (AE Adam, Able Scientific, WA, Australia) and glucose levels were measured using a glucometer (StatStrip Xpress™, Nova Biomedical, Flintshire, UK) previously calibrated with low (ranging 2.6-4.2 mmol/L/46-76 mg/dL), intermediate (ranging 5.0-7.2 mmol/L, 90-130 mg/dL) and high (ranging 14.4-18.3 mmol/L, 260-330 mg/dL) glucose control solutions. 5 µl of blood was collected from a small incision made on the lateral vein of the tail of restrained mouse using a carbon steel surgical blade (Blade size# 15, Swann-Morton, Sheffield, UK) and glycemic readings were recorded from the first blood droplet.
Figure 5: Experimental grouping of NOD.B10 and Balb/c mice at 4 weeks of age according to their genotype and diet.

Figure 6: Experimental time line for NOD.B10 mice from 4 to 12 weeks of age.

Figure 7: Experimental time line for Balb/c mice from 4 to 12 weeks of age.
2.6 Intraperitoneal Glucose Tolerance Test (ipGTT)

One week before harvesting, mice were subjected to intraperitoneal glucose tolerance tests (ipGTT). Mice were fasted for 4 hours from 8 AM until 12 PM, with only water available. Body weights were measured to calculate the glucose injection dose (2 g glucose/kg body weight). Mice were restrained and the tail was heated under a heat lamp during bleeding procedures to induce heat vasodilation. Approximately 60 µL of tail blood was collected from a small incision at the lateral vein of the tail, as previously described, into EDTA rinsed microtubes.

At time 0 of the ipGTT, a bolus of glucose (2 g/kg body weight, 25% glucose solution, Baxter Toongabbie, NSW, Australia) was injected intraperitoneally (IP). Glycaemia levels were checked at times 5, 15, 30, 60, 90 and 120 min. Food was provided to the mice after the conclusion of the experiment. 60 µL of tail blood was collected into EDTA-rinsed microtubes at times 0, 15 and 90 min and later centrifuged at 3000 rpm for 15 min at 4°C (Rotor# SX4750, Allegra®X- 15R centrifuge, Beckman Coulter, CA, USA). Plasma collected was then stored in a -80°C freezer for future analysis.

2.7 Analysis of body fat composition by Dual Energy X-ray Absorptiometry (DEXA)

Assessments of body fat composition were performed on 6 and 12 week old mice of both strains, using dual energy X-ray absorptiometry technique (DEXA). Prior to measurements, the PIXImus machine (Lunar PIXImus II Densitometer machine, GE Medical Systems, Lunar, Madison, WI) was calibrated for quality control with a phantom mouse with known values (Bone mass density (BMD) % CV less than 1%,
BMD calibration factor between 0.8 to 1.2, % fat standard deviation result between -1.0 to +1.0 and a % fat offset value between -5.000 and 5.000).

After 4 hours of fasting to prevent calcified food in the image, each mouse was anesthetized by exposure to 2% isoflurane (cat#05260-05, 100% isoflurane, IsoFlo™, Abbott diagnostics, IL, USA) via inhalation in an induction chamber (0.1 L/min flow rate of 2.5% vaporized isoflurane and 0.2 L/min flow rate of oxygen as the delivery gas for the anesthetic vapour). Body scans were conducted in unconscious mouse placed on the scanner bed in a prone position, with limbs and tail stretched away from the body. Continuous supply of isoflurane-oxygen was provided during the entire duration of the DEXA scan of around 5 min. Lacrimal gel (LacriLube®, Allergan, CA, USA) was applied to the eyes of the mice to prevent dryness due to the vaporized anesthesia. Quantitative analysis on the lean and fat mass content and total % body fat content was obtained and analyzed using PIXImus2 2.10 software.

### 2.8 Blood chemistry, euthanasia and tissue harvesting

#### 2.8.1 Blood sampling

On the day of harvesting, around 60 µL of non-fasting tail blood was collected from conscious mice into EDTA-rinsed microtubes containing 10 µL of 0.5M EDTA solution (Ethylenediaminetetraacetic acid, Cat# E9884, Sigma Aldrich, CA, USA). Plasma from these blood samples were centrifuged at 6000 rpm for 10 min at 4°C (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA) and stored in a -80°C freezer for further measurement of plasma insulin and total cholesterol.
2.8.2 Anesthesia

Following blood collection, mice were anesthetized with a mixed solution of ketamine (20 mg/mL, Troy laboratories, NSW, Australia), xylazine (100 mg/mL, Troy laboratories, NSW, Australia), prepared in saline solution (1:1:8, v:v). The dose of anesthesia given to each mouse was 10 µL per gram body weight and it was injected on the left dorsal lower abdominal region using a 25G needle (0.5 mm X 25 mm, Cat# 301807, BD precision glide™ needle, NJ, USA) and 1 mL tuberculin syringe (Cat# 302100, BD Biosciences, NJ, USA).

This procedure was followed by collection of 1 mL of cardiac blood through cardiac puncture using a 25G needle (0.5 mm X 25 mm, Cat# 301807, BD precision glide™ needle, NJ, USA) and 1 mL tuberculin syringe (Cat# 302100, BD Biosciences, NJ, USA). The collected blood was immediately transferred into a 4 mL BD Vacutainer (Cat#366164, BD UK, Plymouth, UK), kept on ice until later centrifugation at 3000 rpm for 15 min at 4°C (Rotor# SX4750, Allegra®X- 15R centrifuge, Beckman Coulter, CA, USA). Plasma collected from these samples were then stored in a -80°C freezer for further quantification of plasma alanine transaminase (ALT), adiponectin and monocyte chemoattractant protein (MCP-1).

2.8.3 Tissue harvesting

All groups of mice were euthanized by cervical dislocation. The abdominal cavity was opened widely and liver and adipose tissue (subcutaneous, peri-ovarian, mesenteric and brown adipose tissue) were dissected and weighed on a scale (FX-200i, Max= 220 g, d= 0.001 g, A&D company Ltd).
2.8.3.1 Liver dissection

The right lobe of the liver was quickly dissected, weighed and cut into small segments. One part was stored in disposable vinyl specimen molds (Tissue-Tek® Cryomold® standard, 25 mm x 20 mm x 5 mm, Cat# 4557, ProSciTech, QLD, Australia) covered in embedding medium (OCT compound, Cat# IA018, ProSciTech, QLD, Australia), frozen on dry ice and later stored in a -80°C freezer. Another part was placed inside a plastic cassette (Embedding cassette, Cat# LID-05, Techno Plas, SA, Australia), fixed in 10% neutral buffered formalin solution blue (cat# ANBFB, Biostain, VIC, Australia), and embedded in paraffin for later immunohistochemistry analysis. Small portions of the liver were also snap-frozen in 7 mL screw cap tubes (Cat# 58.536, Sarstedt, Germany) and later stored in a -80°C freezer for measurement of total TG content using a commercial kit (refer to item 2.10).

The other lobes of the liver were also dissected, weighed, cut into small parts, snap-frozen and later stored in -80°C freezer.

2.8.3.2 Adipose tissue

Fat pad measurements from 4 different anatomical regions were obtained. Mesenteric WAT was scraped from around the intestines, while peri-ovarian and subcutaneous adipose tissues were dissected from the right side of the mouse. BAT was dissected out of the dorsal adipose tissue, behind the neck of the mouse. All the adipose tissues were quickly weighed and cut into three small sections. The first section was embedded in cryomolds (Tissue-Tek® Cryomold® standard, Cat# 4557, ProSciTech, QLD, Australia) covered by embedding medium (OCT compound, Cat# IA018, ProSciTech, QLD, Australia). The second section was placed in plastic cassettes (Embedding cassette, Cat#
LID-05, Techno Plas, SA, Australia), followed by fixation in 10% formalin solution (cat# ANBFB, Biostain, VIC, Australia) for immunohistochemistry analysis and the third section of the adipose tissue was snap-frozen in 7 mL screw cap tubes (Cat# 58.536, Sarstedt, Germany) and later stored in a -80°C freezer for future mRNA expression analysis.

### 2.9 Blood sample analysis

#### 2.9.1 Radioactive Insulin Assay (RIA)

Quantification of plasma insulin levels were determined by radioimmunoassay. This protocol required incubation of a known fixed concentration of radio-labelled tracer antigen (Cat # MP9011, ¹²⁵I- labelled insulin, Abacus, Millipore, MA, USA), with a known amount of anti-insulin antibody (Cat # ML1013-K, Millipore-Linco cat, Billerica, USA), so that there was limited antigen binding sites on the antibody. When plasma sample was added, it generated a competition between labelled and unlabeled insulin for binding sites of the antibody. Therefore, an increased concentration of unlabeled antigen decreased the ratio of antibody-bound tracer to free tracer and vice versa.

Briefly, a standard curve was generated using purified human insulin (Cat# I-9278, Sigma, USA) diluted in BSA- Phosphate buffer at concentrations of 0, 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8 and 16 ng/mL. 50 µL of standards and plasma samples previously diluted in BSA- Phosphate buffer, were distributed into 12 mm X 75 mm polypropylene tubes (Cat#T400A, Simport, QC, Canada) in duplicates. Addition of 50 µL of ¹²⁵I insulin tracer (Cat# MP9011, Millipore, MA, USA) at <0.143 µCi/mL and 50 µL of
diluted guinea pig anti-rat insulin serum (Cat#ML1013K, Millipore, MA, USA) to these tubes was followed by overnight incubation at 4°C. Four duplicate tubes containing 50 µL of tracer alone were prepared without the addition of guinea pig anti-rat insulin serum to determine the total count and non-specific binding required for data analysis.

On the next day, 500 µL of secondary antibody mixture consisting of 335 µL of polyethylene glycol-sodium phosphate buffer (See Appendix for preparation), 85 µL BSA-sodium phosphate buffer (see Appendix for solution preparation), 40 µL of 1:5 diluted goat anti-guinea pig immunoglobulin G (Equitech-Bio Inc., TX, USA) and 40 µL of 0.057 mg/mL guinea pig serum (Cat# MP1013K, Abacus, Jackson ImmunoResearch Laboratories Inc, PA, USA) were added to all tubes (except total count tubes) and incubated for 2 hours at 4°C. All tubes, except total count tubes, were centrifuged at 2500 rpm (Rotor# SX4750, Allegra®X- 15R centrifuge, Beckman Coulter, CA, USA) for 15 min at 4°C. All the tubes, except total count tubes, were inverted to discard the supernatant and remove any unbound tracer, thus separating the antibody-bound tracer from the free tracer. The quantity of unlabeled antigen (plasma insulin) was determined indirectly by measuring the radioactivity of the retained pellets by counting it at 1 minute/tube using the γ-counter (2480 Automatic gamma counter, WIZARD²®, Perkin Elmer, MA, USA).

2.9.2 Alanine transaminase (ALT)

The amount of alanine transaminase (ALT) present in cardiac plasma samples collected from NOD.B10 and Balb/c mice at various ages was measured using the Abbott Architect c16000 chemical analyzer (Abbott Diagnostics, IL, USA) at the Clinical Chemistry department of ACT Pathology.
2.9.3 Adiponectin

Adiponectin concentrations in cardiac plasma were determined using the Quantikine® ELISA Mouse Adiponectin/Acrp30 Immunoassay (Cat# MRP300, R&D Systems, Minneapolis, MN, USA) according to manufacturer’s instructions. This assay uses the quantitative sandwich enzyme immunoassay technique, where plasma samples containing adiponectin were incubated in a 96-well microplate pre-coated with a monoclonal antibody specific for NS0-expressed recombinant mouse adiponectin protein. Addition of enzyme-linked polyclonal antibody specific for mouse adiponectin to the microplate wells causes binding to any mouse adiponectin that had attached to the antibody pre-coated microplate during the incubation period. Hydrogen peroxide and tetramethylbenzidine addition produced a blue colour. This blue colour is suggested to be proportional to the amount of mouse adiponectin in the well.

Prior to assay performance, plasma samples were diluted 2000-fold in Calibrator Diluent RD5-26 (1X) provided in the kit. Mouse adiponectin standard (lyophilized recombinant mouse adiponectin) was reconstituted in Calibrator Diluent RD5-26 (1X) to produce a stock solution of 10 ng/mL and a 2-fold dilution series in Calibrator Diluent RD5-26 (1X) of 5, 2.5, 1.25, 0.62, 0.31 and 0.16 ng/mL concentrations was prepared. A zero standard of 0 ng/mL adiponectin was created by adding only Calibrator Diluent RD5-26 (1X) without the standard stock solution. A positive control of lyophilized recombinant mouse adiponectin with known concentration range was also assayed.

50 µL of plasma samples, standard dilution series and control was distributed to corresponding wells in the microplate provided by the kit. 50 µL of Assay Diluent RD1W (buffered protein base) was added to all the wells and the plate incubated at
room temperature for 2 hours. Wells were then washed five times with 200 µL of wash buffer (provided by the kit) and 100 µL of mouse adiponectin conjugate (enzyme-linked polyclonal antibody specific for mouse adiponectin) was added to all the wells. The plate was then incubated at room temperature for 1 hour. After this incubation period, the plate was flipped over and any unbound enzyme-antibody reagent was removed by washing five times with 200 µL of wash buffer.

100 µL of substrate solution (containing equal parts of hydrogen peroxide and chromogen (tetramethylbenzidine)) was added to all the wells. The microplate was covered with aluminium foil to protect from light and then incubated at room temperature for 30 min producing a blue colour. 100 µL of stop solution containing diluted hydrochloric acid was added to each well to stop the reaction from producing yellow colour. The optical density of each well was determined within 30 min using a microplate reader (Multiskan Ascent, Thermo Lab systems, USA), reading at 450 nm and then at 540 nm. Optical imperfections in the plate were corrected by subtracting the readings at 540 nm from readings at 450 nm.

2.9.4 Monocyte chemoattractant protein-1 (MCP-1)

MCP-1 concentrations in cardiac plasma was determined by ELISA using Quantikine® ELISA Mouse/Rat CCl2/JE/MCP-1 Immunoassay kit (Cat# MJE00, R&D systems, Minneapolis, MN, USA) according to manufacturer’s instructions. Similar to the adiponeectin ELISA kit (Section 2.9.4), this assay uses the quantitative sandwich enzyme immunoassay technique, where plasma samples containing MCP-1 bind to immobilized antibody (raised against E.coli expressed recombinant mouse MCP-1 factor) present in pre-coated 96 well microplates. Addition of a second polyclonal antibody against mouse
MCP-1 conjugated to horseradish peroxidase leads to binding to the plasma MCP-1 attached to the plate. Finally, presence of this immunocomplex is developed by addition of hydrogen peroxide and chromogen tetramethylbenzidine, producing a blue colour, which is suggestive of the proportion of the amount of MCP-1 in the plasma.

Prior to assay development, plasma samples were diluted 2-fold in Calibrator Diluent RD5-3 (provided by the kit). Lyophilized recombinant mouse MCP-1 was reconstituted in Calibrator Diluent RD5-3 to produce a standard stock solution of 1000 pg/mL. A 2-fold standard dilution series was produced from this stock solution by additional dilution using Calibrator Diluent RD5-3 to produce 500, 250, 125, 62.5, 31.2 and 15.6 pg/mL of mouse MCP-1. A zero standard (0 pg/mL) containing only Calibrator Diluent RD5-3 was added. A positive control of lyophilized recombinant mouse MCP-1 with a known concentration range was also included in the assay.

Briefly, 50 µL of standard dilutions, control and diluted plasma samples were distributed in the 96-well microplate provided by the kit, following addition of 50 µL of assay Diluent RD1W (buffered protein base), and incubation at room temperature for 2 hours. The plate was then washed five times with washing buffer (provided by the kit) to remove any unbound substances in the wells. 100 µL of mouse conjugate (polyclonal antibody against mouse MCP-1 conjugated to horseradish peroxidase) was added to each well following the 2 hour incubation at room temperature. Any unbound conjugate was removed by washing each well 5 times with washing buffer.

100 µL of equal parts of hydrogen peroxide and chromogen (tetramethylbenzidine) was added to each well. The microplate was covered in aluminium foil to protect from light. Following the 30 min incubation at room temperature, which yields a blue colour, 100 µL of stop solution (diluted hydrochloric acid) was added to each well to stop that
reaction, turning the blue colour into yellow. The optical density of each well was determined using a microplate reader (Multiskan Ascent, Thermo Lab systems, USA) at 450 nm and 540 nm. Readings at 540 nm were subtracted from readings at 450 nm to correct for optical imperfections in the plate.

2.10 Total hepatic triacylglyceride (TG) content measurement

Concentration of triacylglycerides (TG) in liver samples was measured by the enzymatic hydrolysis of TG into free fatty acids and glycerol. Briefly, 40-50 mg of liver tissue was homogenized for 10 seconds in the presence of 500 µL of 0.5 M KOH prepared in ethanol (see appendix for preparation) in a 2 mL microtube (Cat# 34127-3317, Edwards Instruments Co, USA) using a tissue homogenizer (Tissue Tearor, 5000 to 32,000 rpm speed, BioSpec products Inc., OK, USA). The tubes were then incubated in a 70°C water bath (Ratek shaking waterbath, VIC, Australia) for 20 min. Samples were allowed to cool down for 10 min at room temperature (20-25°C). 1 mL of 0.15 M MgSO₄ solution (see Appendix for preparation) was added to each sample to solubilize the lipids.

A solution of 500 µL of 0.5 M KOH and 1 mL of 0.15 M MgSO₄ was used as a blank sample. All samples were vortexed (Ratek Vortex mixer, VIC, Australia) and centrifuged at 4750 rpm (Rotor# SX4750, Allegra®X- 15R centrifuge, Beckman Coulter, CA, USA) for 5 min at 4°C. The supernatant was then transferred into a new set of 2 mL microtubes (Cat# 34127-3317, Edwards Instruments Co, USA) and the amount of TG was measured using a Triglyceride Determination kit (Cat# TR0100, Sigma, USA) according to manufacturer’s instructions. This TG colorimetric kit
measures the glycerol released from the enzymatic hydrolysis of TG into free fatty acids and glycerol by the action of lipoprotein lipase (Figure 8).

A standard curve containing 0, 0.052, 0.104, 0.156, 0.208 and 0.26 g/L of glycerol was produced. 10 µL of samples, standards and blank were distributed in duplicates in a 96-well microplate (IWAKI, Cat# 3860-96, Osaka, Japan). 160 µL of freshly prepared Reagent A (free glycerol reagent) was added to each well and the microplate was incubated at room temperature for 15 min. 40 µL of reagent B (TG reagent) was then added to each well, and the microplate was incubated at room temperature for another 15 min, followed by measurement of absorbance at 540 nm using the FLUOstar optima spectrophotometer (BMG LABTECH microplate reader, VIC, Australia).
Figure 8: Scheme of reactions used in a chemical colorimetric assay for quantification of triacylglyceride (TG) in liver tissue. Abbreviations used: 4-AAP: 4-aminoantipyrine; ESPA: N-ethyl-N-(3-sulfoprotyl)-M-anisidine (ESPA).
2.11 Molecular Biology

2.11.1 Peri-ovarian adipose tissue RNA extraction

RNA extraction was performed using Trizol® reagent (Cat # 15596-018, Ambion Life Technologies, CA, USA) according to manufacturer's instructions. Briefly, 50 mg of peri-ovarian WAT was homogenized and emulsified in a 2 mL microtube (Cat# 34127-3317, Edwards Instruments Co, USA) containing 1 mL of ice-cold Trizol® reagent for 10 seconds using a tissue homogenizer (Tissue Tearor, 5000 to 32,000 rpm speed, BioSpec products Inc., OK, USA). The homogenate was incubated at room temperature for 5-10 min. This procedure was followed by centrifugation at 12,000 rpm (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA) for 10 min at 4°C, to separate nucleic acids from cell debris.

The resulting pellet contained extracellular matrix, polysaccharides and high molecular weight DNA, while the supernatant contained the RNA. In samples obtained from a HF-fed mouse, a layer of fat found above the supernatant was removed. The RNA-containing supernatant was transferred to a fresh set of 1.5 mL microtubes (Cat# 72.690.550, Sarstedt, Germany), where 200 µL of 100% (v/v) chloroform (Cat # 27710, BDH Chemicals, QLD, Australia) was added. Microtubes were vortexed vigorously (RateX Vortex mixer, VIC, Australia) and allowed to settle for 15 min at room temperature. Samples were centrifuged at 12,000 rpm for 15 min at 4°C (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA).

The chloroform separated the sample into a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. The upper aqueous phase was around 50 % of the total volume and the RNA remained exclusively in this phase. This phase was transferred into a sterile 1.5 mL microtube (Cat# 72.690.550, Sarstedt, Germany).
without disturbing the interphase. The RNA was then precipitated by the addition of 500 µL 100 % (v/v) isopropanol (Cat# 425, Univar, Ajax FineChem, VIC, Australia) and 20 µL of 5 M NaCl (see Appendix for preparation) to increase RNA yield. The sample was mixed by gentle inversion and equilibration was allowed for either 10 min at room temperature, or 30 min at 4°C. The sample was then centrifuged at 12,000 rpm (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA) for 15 min at 4°C to pellet the RNA and the supernatant was discarded.

The RNA pellet was washed twice by vortexing (Ratex Vortex mixer, VIC, Australia) in 1 mL of ice cold 75% ethanol (see Appendix for preparation), followed by centrifugation at 12,000 rpm (Rotor# SX4750, Allegra X-15R Centrifuge, Beckman Coulter, CA, USA) for 10 min at 4°C. The ethanol was discarded and the samples were placed inside a fume hood (Dyna Safe control system MK3, Dyna Flow, NSW, Australia) to remove excess of ethanol by evaporation. This procedure was performed for no longer than 20 min, to prevent the pellet from drying out, which can cause pellet insolubility. The RNA pellet was resuspended in 20 µL DEPC-water (see Appendix for preparation) and incubated at 55°C in a dry bath incubator (Cat# MD-02W-220, Major Science, Taiwan) for 10 min to allow homogenization. Quantitative and qualitative analysis of the RNA obtained was conducted by spectrophotometry using the Nanodrop spectrophotometer (TM100, ThermoFisher Scientific, USA) and electrophoresis analysis.
2.11.2 Electrophoresis

RNA integrity was analyzed in 1% agarose gel electrophoresis. Sharp and clear 28S and 18S RNA bands indicated intact and high quality RNA, while smeared bands indicated partially degraded and low quality RNA. Briefly, 0.5 g of agarose (Cat# 16103102, BioRad, CA, USA) was dissolved in 50 mL of 1X TAE buffer (see Appendix for preparation), microwaved for 2 min and allowed to cool to approximately 60°C. 2 µL of ethidium bromide at 10 mg/mL (Cat# 161-0433, BioRad, CA, USA) was added to the gel, which was allowed to set for 1 hour in a gel chamber set up with combs. On solidification, the combs were removed and the gel was placed in the electrophoresis tank (BioRad wide mini sub cell GT, Voltage limit 150 VDC, BioRad, CA, USA) and covered over 2-3 mm with 1X TAE buffer (see Appendix for preparation).

Volume corresponding to 1 µg of RNA was resuspended in DEPC-water (see Appendix for preparation) to make up 13 µL solution and 3 µL of loading buffer dye was added per sample. The loading buffer was made up of bromophenol blue and sucrose. Of note, bromophenol blue is negatively charged in neutral buffer and moves in the same direction as the RNA during electrophoresis. This allows visual monitoring of the progress of the gel. Sucrose provides density to the sample, making it easier to load into the wells on the gel. The final 15 µL of the sample solution was loaded into each well and the gel was run at 80 V for 20 min using the Power Pac Universal power supply (500 V/2.5 A/500 W, BioRad, CA, USA). RNA bands were visualized using the Gel Doc XR System PC (BioRad, CA, USA) and analyzed by Vision Capture 14.2 software.
2.11.3 cDNA synthesis

cDNA was synthesized using M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase system (Cat# 28025-01, Invitrogen, USA). Briefly, 1 µg of RNA was resuspended in DEPC-water (see Appendix for preparation) to a final volume of 40.5 µL. 1 µL of random primers Pd (N)₆ (3µg/µL, Cat# 48190-011, Invitrogen, USA) was added to each sample. Samples were incubated at 73°C in a dry bath incubator (Cat# MD-02W-220, Major Science, Taiwan) for 3 min and then placed on ice for 2 min. Samples were then quickly spun and 8.5 µL of reverse transcriptase (RT) master mix was added. The RT master mix was made up of 5 µL 10X RT buffer (refer to Appendix for preparation), 2 µL of dNTP mix at 25 mM (Cat# 10297-018, Invitrogen, USA), 1 µL of 0.5M Dithiothreitol (DTT, Cat# D9779, Sigma Aldrich, CA, USA) and 0.5 µL of 200 U/µL reverse transcriptase M-MLV. Samples were spun and incubated for 1 hour in a 37°C water bath (Ratek shaking waterbath, VIC, Australia) and later stored in a -20°C freezer until use.

2.11.4 Real Time-PCR

Primers used in this study were designed using Gene Runner Software 7/8/8.1 and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and ordered from GeneWorks (VIC, Australia). All primers were resuspended in DEPC-water (see Appendix for preparation) to a final concentration of 100 µM. A working concentration of 10 µM primers was made in DEPC-water. Quantitative Real-Time PCR was performed using the 2X Power SYBR Green Kit (Cat# 4368577, Applied Biosystems, CA, USA) in 5 µL reactions containing 3 µL of mastermix and 2 µL of 1:10 diluted cDNA. The mastermix consisted of 0.5 µL forward primers (0.3 µM), 0.5 µL reverse
Materials and Methods

primers (0.3 µM), 0.2 µL DEPC-water and 2.5 µL of 2X Power SYBR Green. A complete list of the primers (Geneworks, VIC, Australia) and their final concentrations are listed in Table 1.

cDNA samples from each experimental group and mastermix were loaded onto the wells of 384-well clear optical reaction plates (Cat# 128, ABI PRISM, Applied Biosystems, USA) in quadruplicates, using EpMotion 5070 (Eppendorf, Hamburg, Germany) and later sealed with optical film (Applied Biosystems, CA, USA). Gene expression was determined using the ABI PRISM 7900 HT system (Applied Biosystems, CA, USA) under the following conditions: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of 95°C for 15 seconds and primer annealing temperature for 1 min. To validate PCR products, all plates were subjected to dissociation runs at temperature of 95°C for 15 seconds, 60°C for 15 seconds, then slowly ramped 2°C per minute to 95°C for 20 minutes.

The RT-PCR dissociation curves, amplification plot and Ct counts (cycle threshold) were analyzed using SDS 2.4.1 software (Applied Biosystems, CA, USA). Serially diluted pooled peri-ovarian adipose tissue cDNA was used to generate a standard curve for each gene of interest. An XY-scatter graph was plotted to generate a standard curve with Ct values on y-axis and concentration of diluted standards on x-axis. A trendline and linear trendline regression was generated and this equation was used to calculate the expression of each corresponding marker. Six housekeeping genes were tested for stable expression across the different diet and genotype groups in NOD.B10 and Balb/c mice strains, including GAPDH, Cyclophillin, 18S rRNA, HPRT-1, B2M and RPL13a. All results obtained were normalized by dividing their values by the geometric mean of housekeeping genes.
<table>
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<tr>
<th>Gene</th>
<th>Forward (Fwd) primer sequence 5' to 3'</th>
<th>Reverse (Rev) primer sequence 5' to 3'</th>
<th>Annealing Temp (°C)</th>
<th>Product size (bp)</th>
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<td>C/EBP-α</td>
<td>CGG-CGG-GAA-CGC-AAC-AAC-AT</td>
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<td>TGT-CCA-GTG-TCA-GCC-AGA-CTT</td>
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<td>CAA-GGT-GAA-CAG-CTG-GAG-AA</td>
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<td>CAT-TCC-CAC-GAT-TGG-CTA-CA-AGG-AAC</td>
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<td>GCA-CTG-TCA-GAT-TGA-CTG-GAG-AGC</td>
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<td>ACT-CTT-GTG-CCG-TCT-AGT-GTC-AGT-TTC</td>
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<td>CD11c</td>
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<td>GCC-GTC-GGA-GGC-GTT-GGT-TGC-AGG-A</td>
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<td>114</td>
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2.12 Statistical analyses

All analyses were performed using Two-Way analysis of variance (ANOVA) with Bonferroni post hoc testing. A P value of <0.05 was considered significant. Two-Way ANOVA analyses were performed using the PRISM software version 4 (GraphPad Software, San Diego, CA, USA). Data are presented as means ± SEM.
3. Results

3.1 Determination of phenotype- Obesity

Previous long-term studies in HF-fed NOD.B10 foz/foz mice (up to 24 weeks on diet) have shown that the development of T2D and NASH is associated with adipose tissue restriction (attenuation of normal subcutaneous white adipose tissue expansion). If susceptibility of adipose tissue dysfunction (including restriction) in response to nutrient excess, is a primary defect in NOD.B10 mice increasing its susceptibility to T2D and NASH compared to Balb/c mice, it would be expected that differences in the obesity phenotype (including fat distribution), would be apparent between the two strains early on following induction of nutrient overload.

To assess this, body weight, body fat composition scans and mice length analyses over time were performed to evaluate the effects of genotype and diet on the obesity phenotype of these murine models. Therefore, these analyses were conducted in this present study in chow and HF-fed NOD.B10 and Balb/c WT and foz/foz mice.

3.1.1 Fortnightly body weight

Body weight of all NOD.B10 and Balb/c mice were measured every fortnight from 4 weeks of age until the end of the experimental period of 12 weeks of age. Body weight results are shown in Figure 9. Representative photograph of mice showing respective size, shape and intra-abdominal fat content are shown in Figure 10.
The results show that at 4 weeks of age, the body weights of all mice groups were similar within each strain. However, Balb/c mice are smaller than NOD.B10 mice (Figure 9A-B).

HF-feeding did not cause obesity in both WT NOD.B10 and Balb/c mice.

The foz/foz genotype was associated with moderate excessive weight gains in both mice strains. NOD.B10 foz/foz mice on chow diet began to gain more weight than their WT controls from 8 weeks of age, and displayed significant weight gain by 12 weeks of age (36.2 ± 1.0 vs 25.1 ± 0.4 g, foz-chow vs WT-chow, respectively, p<0.0001). Balb/c foz/foz mice fed on chow diet also displayed similar results at 12 weeks of age (30.9 ± 1.7 vs 23.1 ± 0.5 g, foz-chow vs WT-chow, respectively, p<0.001).

The combination of HF-feeding and the foz/foz genotype caused marked obesity in both mice strains. HF-fed NOD.B10 foz/foz mice developed almost 30% more weight gain compared to the chow-fed foz/foz mice at 12 weeks of age (47.9 ± 1.5 vs 36.2 ± 1.0 g, respectively, p<0.0001). Similar results were observed in the Balb/c foz/foz mice at 12 weeks of age (46.7 ± 1.5 vs 30.9 ± 1.7 g, foz-HF vs foz-chow, respectively, p<0.0001).

The initial rate of weight gain tended to be greater in the HF-fed NOD.B10 foz/foz mice compared to the HF-fed Balb/c foz/foz mice. At 6 weeks of age, after only 2 weeks on HF diet, NOD.B10 foz/foz mice displayed higher body weight compared to WT-HF mice (28.4 ± 1.3 vs 22.4 ± 0.6 g, respectively, p<0.001) and foz-chow mice (28.4 ± 1.3 vs 22.7 ± 0.4 g, respectively, p<0.01). This difference in weight gain progressively increased with time, and by 12 weeks of age, HF-fed NOD.B10 foz/foz mice were almost two times heavier than WT-HF mice (47.9 ± 1.5 vs 27.1 ± 1.1 g, respectively, p<0.0001). HF-fed Balb/c foz/foz mice displayed a similar pattern but with progressive increase in body weight from 8 weeks of age, compared to WT-HF mice (30.3 ± 1.0 vs
22.5 ± 0.6 g, respectively, p<0.0001) and foz-chow mice (30.3 ± 1.0 vs 23.2 ± 1.2 g, respectively, p<0.0001).

The rate of weight gain from 10-12 weeks of age slowed in HF-fed NOD.B10 foz/foz mice compared to Balb/c foz/foz mice, such that the earlier differences in weight gain of the two strains was not present at 12 weeks of age (Figure 9C). At 12 weeks of age, HF-fed foz/foz mice from both strains developed similar body weights (47.9 ± 1.5 vs 46.7 ± 1.5 g, NOD.B10 vs Balb/c, respectively).

Taken together, in both NOD.B10 and Balb/c strains, the foz/foz genetic background contributed to the increase in body weight and this was exacerbated when the foz/foz genotype was coupled with HF diet. Both mice strains developed severe obesity by 12 weeks of age, but HF-fed NOD.B10 foz/foz mice initially gained more weight faster than their Balb/c counterparts.
Figure 9: Fortnightly measurements of body weight in female mice. (A) NOD.B10, (B) Balb/c mice strains and (C) comparison between HF-fed foz/foz mice of both strains from 4 to 12 weeks of age. HF-fed foz/foz mice from both strains showed progressive increase in body weight compared to the other groups. Data are means ± SEM of 16-23 mice per group. Two-way ANOVA with Bonferroni post-hoc test; †p<0.05, +++p<0.001, ++++p<0.0001, WT-chow vs foz-chow; ♯♯♯p<0.001, ♯♯♯♯p<0.0001, WT-HF vs foz-HF; ¶¶p<0.001, ¶¶¶¶p<0.0001, foz-chow vs foz-HF; *p<0.05, ****p<0.0001, NOD.B10 vs Balb/c.
Figure 10: Representative photographs showing comparative body shape and size of female WT and \textit{foz/foz} on chow and HF diet of NOD.B10 strain (brown mice) and Balb/c strain (white mice) at 12 weeks of age. The lower panel for each strain shows the relative amount of intra-abdominal adipose tissue.
3.1.2 Measurement of mice body length

Measurement of mice length is a very simple technique employed to analyze differences in growth rate between mice strains. Herein, mice body length (nose to anus) was measured in unconscious NOD.B10 and Balb/c mice on the day of harvesting. NOD.B10 mice lengths were measured at 6, 8, 10 and 12 weeks of age, while Balb/c mice lengths were measured at 6 and 12 weeks of age only. Results are shown in Figure 11.

No significant nose to anus length difference was observed between all groups of mice on the NOD.B10 or Balb/c strains (Figure 11A-B). Mice lengths of all mice groups were also similar when comparing the two mice strains at 6 (data not shown) and 12 weeks of age (Figure 11C).
Figure 11: Body length measurements determined from the tip of the nose to the anus. (A) NOD.B10, (B) Balb/c, and (C) comparisons between strains at 12 weeks of age. No significant difference in mice lengths were observed in any of the mice groups in both strains. Data are means ± SEM of 5-15 mice per group. Two-way ANOVA with Bonferroni post-hoc test. No significant differences found.
3.1.3 Body fat composition (DEXA)

Increases in body weight due to excessive nutrient supply can cause changes in body composition. Thus, using DEXA, we examined fat mass distribution at 6 and 12 weeks of age in all mice groups of NOD.B10 and Balb/c strains fed on chow or HF diet (Figure 12 and 13).

At 6 weeks of age, total lean mass was similar between all mice groups, with the exception of NOD.B10 \(foz/foz\) mice on chow diet, that had more total lean mass than its Balb/c counterparts (16.9 ± 0.7 vs 13.6 ± 0.3 g, respectively, \(p<0.001\)) (Figure 13A). At this same age, only NOD.B10 \(foz/foz\) mice on HF diet higher total fat mass compared to its Balb/c counterparts (5.9 ± 0.6 vs 4.4 ± 0.3 g, respectively, \(p<0.05\)) (Figure 13C). A mild increase in % of body fat mass was observed at 6 weeks of age with HF-feeding in WT and \(foz/foz\) mice of both strains (Figure 13E).

At 12 weeks of age, total lean mass was still very similar between mice groups of both strains, with the exception of HF-fed NOD.B10 \(foz/foz\) mice, which displayed slightly higher total lean mass than its Balb/c counterparts (21.8 ± 0.9 vs 18.1 ± 0.6 g, respectively, \(p<0.05\)) (Figure 13B). At this same age, \(foz/foz\) mice fed on chow diet of both strains, exhibited increased fat mass (shown as absolute terms and as a % of body weight) when compared to their respective WT controls (Figure 13D, F). A further similar increase in fat mass was observed in \(foz/foz\) mice of both strains when fed on a HF diet (14.9 ± 0.4 vs 23.9 ± 2.5 g, NOD.B10 \(foz\)-chow vs \(foz\)-HF, respectively, \(p<0.0001\)) and (10.4 ± 1.9 vs 25.7 ± 1.5 g, Balb/c \(foz\)-chow vs \(foz\)-HF, respectively, \(p<0.0001\)).

In agreement with results obtained from body weight measurements, DEXA body fat composition scans showed that \(foz/foz\) mice of both strains exhibited similar increase in
fat mass content at both ages compared to their WT controls, with even higher fat mass observed when fed on HF diet. Of note, however, at 6 weeks of age, NOD.B10 foz/foz mice had greater fat mass compared to its Balb/c counterparts (Fig 13C), but this was not the case at 12 weeks of age (Fig 13D).

**Summary of results related to development of obesity**

The results show that in response to HF-feeding, NOD.B10 foz/foz mice compared to Balb/c foz/foz mice tend to have a greater initial weight gain and adipose tissue growth. However, this is not sustained out to 12 weeks of age. The results would be consistent with the development of relative adipose restriction in NOD.B10 foz/foz mice at 12 weeks of age. However, these results alone do not prove this, as other explanations such as a relative change in food intake towards 12 weeks of age in HF-fed NOD.B10 foz/foz mice could also cause these results.
Figure 12: Representative body composition scans determined by dual-energy X-ray absorptiometry (DEXA). NOD.B10: (A) WT-chow, (B) WT-HF, (C) foz-chow and (D) foz-HF mice groups and Balb/c: (E) WT-chow, (F) WT-HF, (G) foz-chow and (H) foz-HF mice groups at 12 weeks of age.
Figure 13: Total lean mass (A-B), total fat mass (C-D) and % body fat (E-F) measurements by DEXA of NOD.B10 and Balb/c mice strains at 6 and 12 weeks of age. HF-feeding and foz/foz genotype was observed to cause a progressive increase in fat mass. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, §§p<0.01, §§§p<0.0001, WT-chow vs WT-HF; ++++p<0.0001, WT-chow vs foz-chow; ¶¶¶¶p<0.0001, WT-HF vs foz-HF; ****p<0.0001, foz-chow vs foz-HF; *p<0.05, ***p<0.001, NOD.B10 vs Balb/c.
3.2 Determination of phenotype- Type 2 Diabetes

Previous studies have shown that NOD.B10 foz/foz mice develop T2D by 24 weeks on HF diet.[360] However, the temporal relationship between the development of hyperglycaemia and adipose tissue changes in these mice is unknown. Therefore, serial non-fasting blood glucose levels and plasma insulin levels were measured, as well as intraperitoneal glucose tolerance tests performed to determine the temporal onset of hyperglycaemia and T2D compared to adipose tissue changes in HF-fed NOD.B10 foz/foz mice. The other NOD.B10 and Balb/c mice groups were also assessed for full comparison of phenotypes.

3.2.1 Non-fasting blood glucose levels

Non-fasting blood glucose levels (from tail vein blood) were measured between 9-10 am every fortnight from 4 to 12 weeks of age for all female NOD.B10 and Balb/c mice.

HF-feeding did not cause an increase in blood glucose levels of WT mice of both NOD.B10 and Balb/s strains. These mice remained normoglycaemic throughout the entire study period (Figure 14A).

NOD.B10 foz/foz mice fed on chow diet also maintained normoglycaemia until 12 weeks of age, when a mild elevation in glucose levels were observed compared to WT-chow mice (8.4 ± 0.3 vs 6.8 ± 0.2 mmol/L, respectively, p<0.05).

HF-feeding and the foz/foz genotype in NOD.B10 foz/foz mice caused a progressive increase in glucose levels, initially observed at 8 weeks of age, when compared to foz-chow mice (9.5 ± 0.7 vs 7.9 ± 0.2 mmol/L, respectively, p<0.0001). These values continued to increase over the time course, and by 12 weeks of age, all mice were
hyperglycaemic with 50% of them considered to be diabetic (11.7 ± 0.8 vs 8.4 ± 0.3 mmol/L, foz-HF vs foz-chow, respectively, p<0.0001).

Of note, Balb/c foz/foz mice, fed on either chow or HF diet, remained normoglycaemic throughout the time course (Figure 14B).

Thus, at 12 weeks of age, there was a marked difference in blood glucose levels between HF-fed NOD.B10 foz/foz mice and HF-fed Balb/c foz/foz mice (11.7 ± 0.9 vs 6.9 ± 0.2 mmol/L, respectively, p<0.0001) (Figure 14C).

HF-fed NOD.B10 foz/foz mice developed progressive elevations in non-fasting blood glucose levels, which were evident at a very early age, aggravating with time and leading to the development of diabetes, while HF-fed Balb/c foz/foz mice displayed excellent ability to maintain normoglycaemia. At the age at which blood glucose levels began to increase in HF-fed NOD.B10 foz/foz mice, there was no evidence of adipose tissue restriction.
Figure 14: Time course measurement of non-fasting tail blood glucose levels. (A) NOD.B10 mice, (B) Balb/c mice and (C) comparison between HF-fed foz/foz mice of both strains from 4 to 12 weeks of age. HF-fed NOD.B10 foz/foz mice developed hyperglycemia while Balb/c mice remained normoglycaemic. Data are means ± SEM of 16-23 mice per group. Two-way ANOVA with Bonferroni post-hoc test. +p<0.05, WT-chow vs foz-chow; ###p<0.001, ####p<0.0001, WT-HF vs foz-HF; *****p<0.0001, foz-chow vs foz-HF; **p<0.01, ****p<0.0001, NOD.B10 vs Balb/c.
3.2.2 Non-fasting plasma insulin levels

Overfeeding results in obesity, causing insulin resistance. In order to maintain normoglycaemia, the pancreatic islet β-cells need to compensate for the insulin resistance with insulin hyper-secretion. Failure to sustain this β-cell compensatory response is believed to contribute to hyperglycaemia and the development of T2D.

Therefore, at the harvesting day, non-fasting tail blood was collected from anaesthetized mice for determination of plasma insulin levels from 6 to 12 weeks of age.

HF-feeding did not cause an increase in plasma insulin levels in the WT mice of both strains throughout the study (Figure 15A-B).

The foz/foz genotype caused progressive elevations in plasma insulin levels in the NOD.B10 foz/foz mice fed on chow diet, when compared to WT-chow mice, and by 12 weeks of age, these mice were hyperinsulinaemic (5.1 ± 1.3 vs 0.4 ± 0.1 ng/mL, foz-chow vs WT-chow, respectively, p<0.01). However, the foz/foz genotype did not cause any significant increase in plasma insulin levels in the Balb/c mice strain (Figure 15B).

The combination of HF-feeding and the foz/foz genotype in the NOD.B10 foz/foz mice led to higher plasma insulin levels even at 6 weeks of age, when compared to the foz-chow mice (2.2 ± 0.4 vs 0.8 ± 0.1 ng/mL, respectively, p<0.01). These values augmented progressively until the end of the time course at 12 weeks of age (55.7 ± 9.2 vs 5.1 ± 1.3 ng/mL, foz-HF vs foz-chow, respectively, p<0.0001).

HF-fed Balb/c foz/foz mice developed hyperinsulinaemia at 12 weeks of age, when compared to foz-chow mice (5.3 ± 1.4 vs 1.4 ± 0.3 ng/mL, p<0.0001) and WT-HF mice (5.3 ± 1.4 vs 0.6 ± 0.1 ng/mL, p<0.0001). However, the plasma insulin levels in these
mice were significantly lower than its NOD.B10 counterparts (5.3 ± 1.4 vs 55.7 ± 9.4 ng/mL, respectively, p<0.0001) (Figure 15C).

In the NOD.B10 mice, the $foz/foz$ genotype alone caused hyperinsulinaemia only at 12 weeks of age. However, HF-feeding in these mice caused massive augmentation from 8 weeks of age, leading to profound severe hyperinsulinaemia, with 10 times higher plasma insulin levels than their Balb/c counterparts by the end of the experimental period. Significant hyperinsulinaemia was therefore an early failure in the NOD.B10 mice strain in response to marked overfeeding, as a consequence of the combination of the $foz/foz$ genotype and HF-feeding.
Figure 15: Time course measurement of non-fasting plasma insulin levels. (A) NOD.B10 mice, (B) Balb/c mice and (C) comparison between NOD.B10 and Balb/c HF-fed foz/foz mice from 4 to 12 weeks of age. Data are means ± SEM of 16-23 mice per group. Two-way ANOVA with Bonferroni post-hoc test. $^{****}p<0.0001$, WT-HF vs foz-HF; $^{****}p<0.0001$, foz-chow vs foz-HF; $^{****}p<0.0001$, NOD.B10 vs Balb/c.
3.2.3 Intraperitoneal Glucose Tolerance Test (ipGTT)

To further determine the effects of HF-feeding and the foz/foz genotype on blood glucose regulation and insulin secretion on the two mice strains, ipGTT was performed in all mice groups at 5 and 11 weeks of age, one week prior to harvesting.

At 5 weeks of age, fasting blood glucose levels (time 0 min) were similar between all NOD.B10 (Figure 16A) and Balb/c (Figure 16B) mice groups. As seen in Figure 17A, HF-fed NOD.B10 foz/foz mice developed glucose intolerance when compared to foz-chow mice, particularly at time 30 min (22.8 ± 2.8 vs 15.7 ± 1.8 mM, respectively, p<0.05). These mice also displayed increased fasting plasma insulin levels when compared to its foz-chow controls at baseline (time 0 min) (0.9 ± 0.1 vs 0.5 ± 0.1 mM) and time 90 min (1.3 ± 0.2 vs 0.8 ± 0.1 mM, respectively, p<0.01) (Figure 16C).

All Balb/c mice groups displayed excellent glucose control in response to intraperitoneal glucose load (Figure 16B). This effect was associated with elevations in plasma insulin levels with higher responses in mice fed on HF diet (Figure 16D).

At 11 weeks of age, HF-feeding caused mild glucose intolerance in the NOD.B10 WT mice (Figure 17A). The foz/foz genotype also generated similar results in the NOD.B10 foz/foz mice, with the development of mild hyperinsulinaemia. However, the Balb/c mice did not display these results and remained normoglycaemic (Figure 17B).

Both HF-feeding and the foz/foz genotype led to higher glucose levels even during fasting (at time 0 min) in NOD.B10 foz/foz mice, when compared to foz-chow mice (11.9 ± 0.6 vs 7.9 ± 0.2 mM, respectively, p<0.05), and development of severe glucose intolerance after receiving the intraperitoneal glucose bolus (31.9 ± 1.3 vs 22.4 ± 2.0
mM, respectively, at time 30 min, p<0.001) (Figure 17A). Interestingly, the hyperglycaemia in these mice was associated with severe hyperinsulinaemia (Figure 17C).

HF-fed Balb/c \(foz/foz\) mice displayed a slight increase in glucose levels towards the end of the experiment at time 90 min (15.2 ± 1.9 vs 8.7 ± 0.7 mM, foz-HF vs foz-chow mice, respectively, p<0.05) (Figure 17B). This same group of mice displayed mild hyperinsulinaemia during fasting (time 0 min) (2.2 ± 0.3 vs 0.8 ± 0.2 mM, foz-HF vs foz-chow, respectively) (Figure 17D). Insulin secretion was augmented in response to glucose injection (time 15 min) but it returned close to initial values at time 90 min (2.2 ± 0.3 vs 3.3 ± 0.3 mM, Balb/c foz-HF, time 0 min vs time 90 min, respectively).

After only 1 week on HF diet, at 5 weeks of age, NOD.B10 \(foz/foz\) mice were already displaying signs of impaired glucose tolerance, and by 11 weeks of age, they developed severe glucose intolerance during ipGTT, despite increased plasma insulin levels. Whereas, at 5 weeks of age HF-fed Balb/c \(foz/foz\) mice exhibited normal glucose tolerance maintained by mild elevation in plasma insulin levels, whereas by 11 weeks of age, higher levels of insulin were necessary to maintain a normal glucose response during the test.

**Summary of results relating to development of diabetes**

HF-fed NOD.B10 \(foz/foz\) mice displayed early signs of hyperglycaemia, hyperinsulinaemia and glucose intolerance, followed by development of severe impaired glucose tolerance, despite profound hyperinsulinaemia. On the other hand, HF-fed Balb/c \(foz/foz\) mice maintained normal glucose tolerance and normoglycaemia, accompanied by moderate hyperinsulinaemia. Of note, HF-fed NOD.B10 \(foz/foz\) mice
already had mild hyperglycaemia after only 1 week on the diet at which time insulin levels were not different from the Balb/c counterparts. These results suggest that HF-fed NOD.B10 foz/foz mice are able to sustain very high insulin levels \textit{in vivo}, but the hyperinsulinaemia is inadequate to control blood glucose levels, whereas HF-fed Balb/c foz/foz mice are diabetes resistant.
Figure 16: Intraperitoneal Glucose Tolerance Test (ipGTT) performed on NOD.B10 (left panel) and Balb/c (right panel) mice at 5 weeks of age. Graphics (A, B) and (C, D) depict glucose and insulin excursions during the test, respectively. HF-fed NOD.B10 foz/foz mice developed glucose intolerance during the experiment despite the high plasma insulin levels. HF-fed Balb/c foz/foz mice had higher plasma insulin levels and did not develop hyperglycaemia. Data are means ± SEM of 5-10 mice per group. Two-way ANOVA with Bonferroni post-hoc test.

\[ p<0.05, \text{WT-HF vs foz-HF}; p<0.05, p<0.01, \text{foz-chow vs foz-HF}. \]
Figure 17: Intraperitoneal Glucose Tolerance Test (ipGTT) performed on NOD.B10 (left panel) and Balb/c (right panel) mice at 11 weeks of age. Graphics (A, B) and (C, D) depict glucose and insulin excursions during the test, respectively. HF-fed NOD.B10 \textit{foz/foz} mice developed glucose intolerance despite the high plasma insulin levels. HF-fed \textit{foz/foz} Balb/c mice had higher plasma insulin levels and only developed hyperglycemia towards the end of the experiment. Data are means ± SEM of 5-10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. $^+$ $p<0.001$, $^{+++}p<0.0001$, WT-chow vs foz-chow; $^\#p<0.05$, $^{##}p<0.01$, $^{###}p<0.001$, WT-HF vs foz-HF; $^*p<0.05$, $^{**}p<0.01$, foz-chow vs foz-HF.
3.3 Determination of phenotype- Liver disease

Female NOD.B10 foz/foz mice have been found to develop hepatomegaly as well as increased intrahepatic TG content and elevated plasma ALT levels associated with NASH within 24 weeks on HF diet. The increase in plasma ALT levels is a marker for hepatic injury and transition of NAFLD to NASH. Therefore, liver weight, hepatic TG content and plasma ALT levels were analyzed in foz/foz mice of both NOD.B10 and Balb/c strains from 6 to 12 weeks of age in order to determine the time of transition of NAFLD to NASH. This could then be related to the timing of changes in glucose regulation and adipose tissue parameters.

3.3.1 Liver weight

Total liver tissue weights expressed as absolute weight and as % of body weight were measured at different ages in both mice strains (Figure 18, left and right panels, respectively).

In the NOD.B10 mice, all mice groups had similar liver weights at 6 weeks of age (Figure 18A-B). HF-feeding did not cause any further increase in liver weight in the WT mice throughout the time course. The foz/foz genotype initially caused slightly elevated liver weights in chow-fed NOD.B10 foz/foz mice when compared to the WT controls. However, no further changes were observed after the initial increase in liver weight.

Interestingly, HF-feeding and the foz/foz genotype both caused a marked increase in liver weight in the NOD.B10 foz/foz mice from 10 weeks of age, when compared to foz-chow mice, and when normalized to a % of body weight it still showed marked
hepatomegaly (6.7 ± 0.3 vs 4.8 ± 0.1, as % body weight, p<0.0001) (Figure 18B). This difference in liver weight was progressive with time and by 12 weeks of age, these mice developed progressive hepatomegaly when compared to their foz-chow littermates (8.0 ± 0.3 vs 4.4 ± 0.1, as % of body weight, p<0.0001) (Figure 18B).

In the Balb/c mice, all mice groups displayed similar liver weights at 6 weeks of age, similar to the NOD.B10 mice (Figure 18C-D). HF-feeding did not cause any changes in liver weight in the WT mice. The foz/foz genotype also led to similar results in the chow-fed foz/foz mice. In fact, a slight reduction in % of liver weight was observed in these mice, when compared to WT-chow controls (4.3 ± 0.1 vs 5.5 ± 0.1, as % of body weight, respectively, p<0.01) (Figure 18D).

The combination of HF-feeding and the foz/foz genotype in the Balb/c mice strain led to a significant increase in total absolute liver weight at 12 weeks of age, when compared to foz-chow mice (2.5 ± 0.1 vs 1.2 ± 0.1 g, respectively p<0.001) and WT-HF mice (2.5 ± 0.1 vs 1.4 ± 0.1 g, respectively, p<0.01) (Figure 18C). However, when normalized to a % of body weight, no differences in liver weight were observed (Figure 18D).

Comparison of all mice groups at 12 weeks of age of both strains showed that only HF-fed foz/foz mice on the NOD.B10 background had heavier livers compared to Balb/c mice (7.9 ± 0.3 vs 5.3 ± 0.2, as % of body weight, p<0.001) (Figure 18E-F).

In summary, only HF-fed NOD.B10 foz/foz mice developed significant hepatomegaly by 12 weeks of age, while HF-fed Balb/c foz/foz mice only displayed increase in absolute liver weight which was proportional to body weight gain. Of note, the development of hepatomegaly in the HF-fed NOD.B10 foz/foz mice, as % of body weight, was evident as early as 8 weeks of age.
Figure 18: Liver weight measurements. Total liver weight (left panel) and liver weight normalized to body weight (right panel) of (A-B) NOD.B10 and (C-D) Balb/c mice strains and (E-F) comparison between the 2 strains at 12 weeks of age. Data are means ± SEM of 10-23 mice per group. Two-way ANOVA with Bonferroni post-hoc test. ++p<0.01, WT-chow vs foz-chow; ♯p<0.05, ♯♯♯♯p<0.0001, WT-HF vs foz-HF; ¶¶¶p<0.001, ¶¶¶¶p<0.0001, foz-chow vs foz-HF; ****p<0.0001, NOD.B10 vs Balb/c.
3.3.2 Total hepatic triacylglyceride (TG) deposition

Previous studies in female NOD.B10 foz/foz mice fed with 24 weeks of HF diet had shown that excessive nutrient stress causes abnormalities in body lipid partitioning leading to ectopic fat deposition, particularly in the liver. Development of steatosis followed by hepatocyte ballooning were observed in these mice. Herein, we studied the temporal development of hepatic steatosis in the NOD.B10 and Balb/c mice groups. Hepatic TG expressed as absolute values per gram of tissue (left panels) or as total TG content per liver (right panels) were obtained for all mice groups (Figure 19).

In the NOD.B10 mice, HF-feeding had already caused an increase in hepatic TG deposition at 6 weeks of age (Figure 19A-B). HF-fed NOD.B10 WT mice displayed higher TG content at 6 weeks of age compared to its WT-chow controls (306.1 ± 39.8 vs 68.9 ± 6.2 mg TG/g w:w, respectively, p<0.0001) and maintained this difference even at 12 weeks of age (Figure 19B). The foz/foz genotype also caused a slight elevation in hepatic TG content in the NOD.B10 foz/foz mice when compared to its WT-chow controls at 12 weeks of age (226.9 ± 32.4 vs 68.9 ± 6.2 mg TG/g w:w, respectively, p<0.0001) (Figure 19B).

The combination of both HF-diet and the foz/foz genotype in the NOD.B10 foz/foz mice was associated with an increase in hepatic TG deposition when compared to the foz-chow mice, observed from 8 weeks of age (971.7 ± 55.8 vs 178.3 ± 32.1 mg TG/g w:w, p<0.0001), which progressively increased with time (Figure 19B).

In the Balb/c mice, HF-feeding was observed to cause similar increase in hepatic TG content in the WT mice, similar to the NOD.B10 WT mice (Figure 19C-D). The foz/foz genotype did not cause any changes in TG deposition in the Balb/c foz/foz mice.
However, HF-feeding with the $foz/foz$ genotype in the Balb/c $foz/foz$ mice led to a two-fold increase in TG deposition at 12 weeks of age.

Comparisons of hepatic TG levels between the two mice strains at 12 weeks of age showed that HF-feeding alone caused significant increased TG levels in both WT and $foz/foz$ mice (Figure 19E-F). However, the $foz/foz$ genotype and HF diet both caused more severe accumulation of hepatic TG in both strains. Interestingly, when these results were expressed as total TG content in the entire liver, a significant difference in TG levels was observed between HF-fed NOD.B10 and Balb/c $foz/foz$ mice ($1229.2 \pm 102.4$ vs $867.0 \pm 38.1$ mg TG/g w:w, respectively, $p<0.0001$) (Figure 19F).

In summary, HF diet seems to drive the ectopic deposition of TG in the liver. since in both mice strains, WT and $foz/foz$ mice showed higher hepatic TG deposition. However, the $foz/foz$ genotype aggravates this by causing three times more TG deposition than its control. Although total hepatic TG per gram of liver tissue was similar in the HF-fed $foz/foz$ mice of both strains, total TG content in the entire liver was higher in HF-fed NOD.B10 $foz/foz$ mice.
Figure 19: Hepatic triacylglyceride (TG) accumulation in NOD.B10 and Balb/c mice fed on chow or HF diet. (A-B) NOD.B10, (C-D) Balb/c and (E-F) comparison between the two strains at 12 weeks of age. Increased hepatic TG deposition was observed with HF-feeding and foz/foz genotype in both strains. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, §§p<0.01, §§§p<0.001, §§§§p<0.0001, WT-chow vs WT-HF; +p<0.05, +++p<0.001, WT-chow vs foz-chow; $p<0.0001, WT-HF vs foz-HF; §§§§p<0.0001, foz-chow vs foz-HF; *p<0.05, ****p<0.0001, NOD.B10 vs Balb/c.
3.3.3 Plasma alanine transaminase (ALT) levels

Increased levels of blood ALT has been extensively used as a marker of hepatocellular liver injury. ALT is a cytoplasmic enzyme that catalyzes the deamination and transamination of amino acids, facilitating crucial metabolic liver functions. Hepatocellular demise causes release of this enzyme into the circulation where it can be measured. Elevated plasma ALT levels can also be used to diagnose the development of NASH. Plasma obtained from cardiac blood during harvesting was used to measure ALT levels in NOD.B10 and Balb/c mice (Figure 20).

In the NOD.B10 mice, at 6 weeks of age, no significant differences in plasma ALT levels were found between the mice groups (Figure 20A). However, by 12 weeks of age, plasma ALT levels in HF-fed NOD.B10 foz/foz mice increased five-fold, compared to the foz-chow mice (267.5 ± 23.1 vs 45.3 ± 4.3 μg/mL, respectively, p<0.0001).

In the Balb/c mice, all mice groups exhibited similar plasma ALT levels at 6 weeks of age (Figure 20B). However, at 12 weeks of age, HF-fed Balb/c foz/foz mice displayed a moderate increase in plasma ALT levels compared to the foz-chow mice (122.1 ± 15.1 vs 55.5 ± 10.6 μg/mL, respectively, p<0.05).

Comparisons between both mice strains at 12 weeks of age showed that HF-fed NOD.B10 foz/foz mice had double the plasma ALT levels than their Balb/c counterparts (267.5 ± 23.1 vs 122.1 ± 15.1 μg/mL, respectively, p<0.0001) (Figure 20C).
Summary of results relating to development of liver disease

The finding that HF-fed NOD.B10 foz/foz mice are the only mouse group that develops disproportionate hepatomegaly and that it develops this early, in association with increased hepatic TG content, would be consistent with early repartitioning of body fat to liver from adipose tissue stores in this strain. The Balb/c foz/foz mice, however, do accommodate more hepatic TG with HF-feeding, but not to the same degree as in the NOD.B10 strain. The ALT results suggest that the increased TG accumulation in the HF-fed NOD.B10 foz/foz mice is associated with elevated ALT levels consistent with the induction of NASH. ALT levels do rise in the HF-fed Balb/c foz/foz mice, but to a much lesser extent.
Figure 20: Alanine transaminase (ALT) levels determined from plasma of non-fasting NOD.B10 and Balb/c strains. (A) NOD.B10, (B) Balb/c and (C) comparisons between mice groups of both strains at 12 weeks of age. At 12 weeks of age, NOD.B10 foz/fz mice fed on HF-diet exhibit higher levels of plasma ALT, indicating the presence of hepatocellular injury. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. 

♯ p<0.05, ♯♯♯♯ ♯ p<0.0001, WT-HF vs foz-HF; ♯♯♯♯ ♯ p<0.0001, foz-chow vs foz-HF; **** p<0.0001, NOD.B10 vs Balb/c.
3.4 Determination of phenotype: Adipose tissue restriction

Failure of safe partitioning of excess nutrients to subcutaneous adipose tissue depots has been implicated in the pathogenesis of NASH. This results in abnormal expansion of visceral adipose tissue, associated with reduced insulin sensitivity and WAT inflammation, and ectopic hepatic fat deposition causing the steatosis of NAFLD. Previous studies on NOD.B10 $foz/foz$ mice have shown an attenuation in normal adipose tissue expansion (adipose restriction) after 6 weeks on HF diet, whereby further adipose tissue expansion did not occur despite increased nutrient intake. In this study, by 12 weeks of age, HF-fed NOD.B10 $foz/foz$ mice developed hepatomegaly, increased hepatic TG deposition and NASH, suggesting adipose restriction. The body weight and body composition data presented in Section 3.1 of the results are also consistent with some development of adipose restriction by about 10 weeks of age in these mice.

Therefore, fad pad measurement of subcutaneous and visceral (mesenteric and periovarian) adipose tissues in both mice strains from 6 to 12 weeks of age, were analyzed to further confirm and characterize the development of adipose restriction.

3.4.1 Subcutaneous WAT expansion

Subcutaneous adipose tissue (SAT) from the right abdominal side of the mice were extracted, weighed, and presented as absolute tissue weights (left panel) and normalized to % of body weight (right panel) (Figure 21). Both analyses displayed similar patterns. In the NOD.B10 mice, absolute SAT weights were similar for all mice groups at 6 weeks of age (Figure 21A). HF-feeding was observed to cause a slightly higher SAT
expansion in WT mice compared to their chow controls. However, the foz/foz genotype was found to cause increased adiposity in the NOD.B10 mice during the time course of the study. Further augmentation in SAT expansion was observed in the NOD.B10 mice when the foz/foz genotype was combined with HF diet. At 6 weeks of age, HF-fed NOD.B10 foz/foz mice already displayed increased adiposity compared to its chow controls (1.3 ± 0.1 vs 0.7 ± 0.1, as a % of body weight, p<0.05) (Figure 21B). By 12 weeks of age, these mice had developed significantly higher SAT weights when compared to foz-chow mice (3.2 ± 0.1 vs 2.6 ± 0.1, as a % of body weight, respectively, p<0.01) (Figure 21B).

In the Balb/c WT mice, a similar pattern of SAT weights as the NOD.B10 WT mice was observed (Figure 21C). However, the adiposity caused by the foz/foz genotype alone was lower in the Balb/c mice when compared to the NOD.B10 mice. A similar pattern of progressive increase in adiposity was observed in the HF-fed Balb/c foz/foz mice when compared to the foz-chow mice at 6 weeks of age (1.5 ± 0.1 vs 0.6 ± 0.1, as a % of body weight, respectively, p<0.0001) and 12 weeks of age (2.7 ± 0.1 vs 1.7 ± 0.1, as a % of body weight, respectively, p<0.0001) (Figure 21D).

Comparison of the increase in adiposity of HF-fed foz/foz mice in both strains suggested that at 12 weeks of age, the NOD.B10 mice had significantly higher adipose tissue expansion than their Balb/c counterparts (3.2 ± 0.1 vs 2.8 ± 0.1, as a % of body weight, p<0.01) (Figure 21F).

In summary, subcutaneous adipose tissue was progressively expanding normally in the HF-fed foz/foz mice of both strains without any signs of adipose restriction.
Figure 21: Subcutaneous WAT accumulation in mice fed experimental diets from 4 to 12 weeks of age. Absolute tissue weights (left panel) and tissue reported as % body weight (right panel) of (A-B) NOD.B10, (C-D) Balb/c and (E-F) comparison between the HF-fed foz/foz mice of both strains. Similar progressive adipose expansion in the HF-fed foz/foz mice of both strains was observed until 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. $$$p<0.001, $$$$p<0.0001$, WT-chow vs WT-HF; $^*$$p<0.05, $^{+++}$$p<0.0001$, WT-chow vs foz-chow; $^{+++}$$p<0.001, $^^^^$$p<0.0001$, WT-HF vs foz-HF; $^\dagger$$p<0.05, $^{\dagger\dagger}$$p<0.01, $^{\dagger\dagger\dagger}$$p<0.001, $^{\dagger\dagger\dagger\dagger}$$p<0.0001$, foz-chow vs foz-HF; $^*$$p<0.05$, NOD.B10 vs Balb/c.
3.4.2 Mesenteric WAT expansion

Mesenteric WAT around the small intestine were extracted, weighed and presented as absolute weights (left panel) and relative to % of body weight (right panel) in Figure 22. NOD.B10 mice from all mice groups displayed similar absolute mesenteric WAT weights at 6 weeks of age (Figure 22A). Interestingly, when these values were expressed as % of body weight, HF-fed NOD.B10 foz/foz mice exhibited higher accumulation of mesenteric fat compared to the controls. HF-feeding in the WT mice was observed to cause a slight tendency to accumulate more mesenteric fat than the WT-chow controls (Figure 22A-B). The foz/foz genotype on the NOD.B10 strain caused progressive augmented mesenteric WAT expansion compared to the WT mice throughout the time course (Figure 22A-B). Furthermore, the foz/foz mice fed on HF-diet developed even further significant increase in adiposity when compared to their chow-fed foz/foz mice controls from 6 weeks of age (1.3 ± 0.1 vs 0.6 ± 0.1, as % of body weight, p<0.01) to 12 weeks of age (3.2 ± 0.1 vs 2.4 ± 0.1, as % of body weight, p<0.001) (Figure 22B).

Balb/c WT mice displayed a similar pattern of mesenteric fat expansion as the NOD.B10 mice (Figure 22C). Chow-fed Balb/c foz/foz mice exhibited a progressive increase in mesenteric adipose tissue which was significantly higher than its WT-chow controls (Figure 22C-D), but similar to the WT-HF mice. Similar to the NOD.B10 mice, by 12 weeks of age, HF-fed Balb/c foz/foz mice developed higher adiposity than chow-fed foz/foz mice (3.7 ± 0.2 vs 2.6 ± 0.1, as % of body weight, p<0.0001) (Figure 22D).

Comparison of the mesenteric WAT weights of HF-fed foz/foz mice of both strains showed that at 12 weeks of age, both mice strains had similar absolute mesenteric WAT weights, but normalization to body weight gain suggest that the Balb/c mice had higher
mesenteric fat expansion than its NOD.B10 counterparts (3.7 ± 0.2 vs 3.2 ± 0.1, as % of body weight, respectively, p<0.05) (Figure 22E-F).

Taken together, these results suggest that mesenteric WAT was expanding normally in the HF-fed foz/foz mice of both NOD.B10 and Balb/c strains, similar to subcutaneous WAT expansion. However, accumulation of mesenteric fat appeared to be less in HF-fed NOD.B10 foz/foz mice compared to HF-fed Balb/c foz/foz mice.
Figure 22: Right mesenteric white adipose tissue weights. Absolute weights (left panel) and tissue weight normalized to body weight (right panel) of (A-B) NOD.B10, (C-D) Balb/c mice and (E-F) comparison between HF-fed foz/foz mice of both strains. Progressive adipose expansion in the HF-fed foz/foz mice of both strains was observed until 12 weeks of age. Values are the means ± SEM of 16-23 mice from in each group. Two-way ANOVA with Bonferroni post-hoc test. §§p<0.01, §§§p<0.001, WT-chow vs WT-HF; ++++p<0.0001, WT-chow vs foz-chow; +++p<0.001, ++++p<0.0001, WT-HF vs foz-HF; *p<0.05, NOD.B10 vs Balb/c.
### 3.4.3 Peri-ovarian WAT expansion

Peri-ovarian adipose tissue from the right side of the mice was extracted, weighed and presented as absolute tissue weights (left panel) and normalized to % of body weight (right panel) at different ages in both NOD.B10 and Balb/c mice strains (Figure 23).

All NOD.B10 mice groups exhibited similar absolute peri-ovarian WAT weights at 6 weeks of age (Figure 23A). Upon normalization to % of body weight, it was observed that HF-feeding caused a tendency to increased peri-ovarian adiposity in these mice compared to chow controls from 6 weeks of age (Figure 23B). However, this increase in adipose weight was not significant and comparatively lower in the WT mice compared to the foz/foz mice. The foz/foz genotype in the NOD.B10 foz/foz mice led to a progressive increase in peri-ovarian adipose expansion throughout the time course (Figure 23A-B) and by 12 weeks of age, adipose tissue weights of these mice were significantly higher than the WT-chow controls (2.5 ± 0.1 vs 0.6 ± 0.1, as % of body weight, p<0.0001) (Figure 23B).

The combination of HF diet and the foz/foz genotype in the NOD.B10 foz/foz mice triggered a significantly higher adipose tissue expansion, and at 12 weeks of age, these mice had double the adiposity than the WT-HF mice (3.9 ± 0.3 vs 1.5 ± 0.1, as % of body weight, p<0.0001) and foz-chow mice (3.9 ± 0.3 vs 2.8 ± 0.2, as % of body weight, p<0.0001) (Figure 23B).

Balb/c WT and foz/foz mice displayed a similar pattern of progressive peri-ovarian adipose tissue expansion similar to its NOD.B10 counterparts (Figure 23C-D). HF-feeding and the foz/foz genotype in the Balb/c foz/foz mice caused higher peri-ovarian adiposity at 12 weeks of age, when compared to WT-HF mice (3.9 ± 0.3 vs 1.5 ± 0.1, as
% of body weight, \( p<0.001 \) and foz-chow mice (3.9 ± 0.3 vs 1.5 ± 0.1, as % of body weight, \( p<0.0001 \)) (Figure 23D).

Comparison of the increase in adiposity of HF-fed \( foz/foz \) mice of both strains suggests no difference in peri-ovarian adipose expansion between both strains (Figure 23E-F).

Taken together, peri-ovarian adipose tissue was found to be progressively expanding similarly in HF-fed \( foz/foz \) mice of both strains throughout the study period.

**Summary of results relating to development of adipose restriction**

Fat pad measurements of subcutaneous WAT and visceral WAT depots (mesenteric and peri-ovarian) suggested that in response to HF-feeding, \( foz/foz \) mice of both NOD.B10 and Balb/c strains had very similar adipose tissue expansion. Contrary to what was predicted, if there is adipose restriction in the HF-fed NOD.B10 \( foz/foz \) mice, it is more likely to be in the mesenteric WAT rather than in the subcutaneous WAT.
Figure 23: Peri-ovarian WAT expansion from 6 until 12 weeks of age. Absolute tissue weight (left panel) and tissue weight expressed as % body weight (right panel) in (A-B) NOD.B10, (C-D) Balb/c mice and (E-F) comparison between HF-fed foz/foz mice of both strains. Progressive adipose tissue expansion was observed in the HF-fed foz/foz mice of both strains until 12 weeks of age. Values are the means ± SEM of 6-23 mice from each group. Two-way ANOVA with Bonferroni post-hoc test. §§p<0.01, WT-chow vs WT-HF; +++p<0.001, ++++p<0.0001, WT-chow vs foz-chow; ♯♯p<0.01, ♯♯♯p<0.001, ♯♯♯♯p<0.0001, WT-HF vs foz-HF; ¶p<0.05, ¶¶p<0.01, ¶¶¶p<0.001, ¶¶¶¶p<0.0001, foz-chow vs foz-HF.
3.4.4 Brown adipose tissue (BAT) measurements

Since BAT activity is inversely proportional to obesity, BAT from the dorsal adipose tissue was extracted, weighed and presented as absolute tissue weight (left panel) and normalized to % body weight (right panel) from NOD.B10 and Balb/c mice to determine any obesity-induced changes in BAT depots (Figure 24).

All NOD.B10 mice groups displayed similar BAT weights at 6 weeks of age (Figure 24A-B). HF-feeding did not induce BAT expansion in WT mice. The foz/foz genotype caused a progressive increase in BAT weight in NOD.B10 foz/foz mice at 12 weeks of age, compared to WT-chow mice (0.5 ± 0.03 vs 0.2 ± 0.01, as % of body weight, p<0.0001) (Figure 24B). The combination of HF-feeding and the foz/foz genotype in the NOD.B10 foz/foz mice led to significantly higher BAT expansion, compared to WT-HF mice at 12 weeks of age (0.5 ± 0.02 vs 0.3 ± 0.02, as % of body weight, p<0.0001) (Figure 24B). However, when normalized to % of body weight, NOD.B10 foz/foz mice on both chow and HF diets displayed similar BAT expansion (Figure 24B).

Balb/c WT mice exhibited similar BAT expansion as the NOD.B10 counterparts (Figure 24C-D). However, unlike the NOD.B10 mice, BAT expansion in chow-fed Balb/c foz/foz mice was less than the HF-fed foz/foz mice and almost similar to the WT-HF mice. HF-fed Balb/c foz/foz mice displayed significantly higher BAT expansion than the chow-fed foz/foz mice at 12 weeks of age (0.5 ± 0.02 vs 0.4 ± 0.02, as % of body weight, p<0.01) (Figure 24D).

Comparison of BAT weight in HF-fed foz/foz mice of both strains showed no inter-strain difference in BAT expansion (Figure 24E-F). In summary, unlike results observed in literature where BAT is inversely correlated with BMI, the HF-fed foz/foz mice of both strains displayed similar BAT expansion, despite the presence of obesity.
Results

Figure 24: Brown adipose tissue weights. Absolute weights (left panel) and tissue weight relative to body weight (right panel) of (A-B) NOD.B10, (C-D) Balb/c mice and (E-F) comparison between the HF-fed foz/foz mice of both strains. Mice with the foz/foz genotype were found to have more brown adipose tissue than the WT mice. Data are means ± SEM of 6-23 mice per group. Two-way ANOVA with Bonferroni post-hoc test. ++p<0.01, +++p<0.001, ++++p<0.0001, WT-chow vs foz-chow; ###p<0.001, ####p<0.0001, WT-HF vs foz-HF; *p<0.05, **p<0.01, foz-chow vs foz-HF.
3.5 Determination of changes in circulating plasma adipokine profile

Adipose tissue dysfunction is associated with altered adipokine profiles, and previous studies on the NOD.B10 foz/foz mice have shown that after 24 weeks on HF diet, these mice develop hypoadiponectinemia and increased serum MCP-1 levels.\cite{360} Therefore, plasma adiponectin and MCP-1 levels were analyzed to determine any changes in secretion of these adipokines and how this relates temporally with changes in glucose regulation, hepatic changes and other parameters of adipose function/dysfunction.

### 3.5.1 Analysis of plasma adiponectin

Plasma obtained from cardiac blood during harvesting was used to measure circulating adiponectin levels in both NOD.B10 and Balb/c mice.

NOD.B10 mice exhibited no difference in plasma adiponectin levels in any mice group at 6 weeks of age (Figure 25A). HF-feeding did not affect adiponectin levels in the WT mice compared to chow-fed control mice (10.8 ± 0.4 vs 11.6 ± 0.4 μg/mL, respectively). The foz/foz genotype also did not cause any alteration in adiponectin levels in NOD.B10 foz/foz mice when compared to WT-chow mice (11.1 ± 0.9 vs 10.8 ± 0.4 μg/mL, respectively). However, the combination of HF-feeding and the foz/foz genotype in the NOD.B10 foz/foz mice caused a progressive drop in plasma adiponectin levels after 8 weeks of age and by 12 weeks of age, these mice had significantly reduced adiponectin levels compared to foz-chow mice (8.3 ± 0.5 vs 11.1 ± 0.9 μg/mL, respectively, p<0.01).

Balb/c mice did not display any difference in plasma adiponectin levels in any mice groups at both 6 and 12 weeks of age (Figure 25B). HF-fed Balb/c foz/foz mice
exhibited a slight tendency to reduce adiponectin levels. However, this reduction was not significant.

At 12 weeks of age, no inter-strain differences in adiponectin levels were observed in HF-fed $f_{o/o}$ mice (Figure 25C).

In summary, HF-fed NOD.B10 $f_{o/o}$ mice developed hypoadiponectinemia at 12 weeks of age compared to its genotype and diet controls. On the other hand, although HF-fed Balb/c $f_{o/o}$ mice did not exhibit a significant decrease in adiponectin levels to its WT and diet controls, at 12 weeks of age, it displayed similar reduced adiponectin levels as its NOD.B10 counterparts. This suggests that both NOD.B10 and Balb/c mice fed on HF diet do not differ with respect to circulating adiponectin levels. This is against the hypothesis that differential susceptibility of NOD.B10 mice to excess energy supply is an early or primary defect in the unhealthy obesity that can be induced in the NOD.B10 strain.
Figure 25: Plasma adiponectin levels from WT and foz/foz mice fed on chow or HF diet from 4 to 12 weeks of age. (A) NOD.B10, (B) Balb/c strains and (C) comparison between the two strains at 12 weeks of age. Plasma adiponectin levels decreased in foz/foz mice on HF-diet of NOD.B10 and Balb/c strains at 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. ###p<0.001, WT-HF vs foz-HF; ##p<0.01, ¶¶¶p<0.001, foz-chow vs foz-HF.
3.5.2 Analysis of Plasma Monocyte Chemoattractant Protein-1 (MCP-1)

MCP-1 is secreted by various cells and is responsible for recruitment and infiltration of monocytes into active areas of inflammation. Elevated MCP-1 levels are often used as a marker for inflammation. Therefore, circulating MCP-1 levels were measured from cardiac blood plasma obtained from NOD.B10 and Balb/c mice on the day of harvesting at 6 and 12 weeks of age for detection of inflammation.

At 6 weeks of age, no significant difference in plasma MCP-1 levels was observed between NOD.B10 and Balb/c strains among any mice groups (Figure 26A).

At 12 weeks of age, a significant increase in MCP-1 levels was observed with HF-feeding in NOD.B10 WT and foz/foz mice, which exhibited markedly higher levels than their Balb/c counterparts (77.7 ± 20.9 vs 25.6 ± 5.1 pg/mL, WT-HF NOD.B10 vs Balb/c, p<0.05) and (154.2 ± 31.4 vs 97.1 ± 15.5 pg/mL, foz-HF NOD.B10 vs Balb/c, p<0.05) (Figure 26B). HF-fed Balb/c foz/foz mice also displayed an increased plasma MCP-1 level, which was however, lower than its NOD.B10 counterpart.

In summary, plasma MCP-1 levels were increased in the HF-fed foz/foz mice of both strains at 12 weeks of age, but the NOD.B10 mice displayed higher circulating plasma MCP-1 levels than its Balb/c counterparts.

Summary of results related to altered adipokine profile

Only HF-fed NOD.B10 foz/foz mice displayed significantly reduced plasma adiponectin levels by 12 weeks of age. However, a similar non-significant reduction was observed in the HF-fed Balb/c foz/foz mice. Plasma MCP-1 level was increased in both these mice groups, suggesting the presence of inflammation. However, HF-fed NOD.B10
*foz/foz* mice exhibited higher plasma MCP-1 levels, which may imply that these mice develop higher degree of inflammation compared to the Balb/c mice. Without other measurements, however, the site of this increase in inflammation is uncertain. It could be increased inflammation of adipose tissue, but NASH could also be a source of increased plasma MCP-1.
Figure 26: Plasma monocyte chemoattractant protein-1 (MCP-1) levels of NOD.B10 and Balb/c mice strains at (A) 6 and (B) 12 weeks of age. HF-fed foz/foz mice of both strains display elevated plasma MCP-1 levels at 12 weeks of age. However, the plasma MCP-1 levels were higher in HF-fed foz/foz mice on the NOD.B10 background. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. ###p<0.0001, WT-HF vs foz-HF; ####p<0.0001, foz-chow vs foz-HF; *p<0.05, NOD.B10 vs Balb/c.
3.6 Peri-ovarian adipose tissue gene expression analysis

The preceding results do not equivocally indicate the presence or absence of adipose restriction and/or dysfunction as early abnormalities in the NOD.B10 mice compared to the Balb/c mice, in response to excessive nutrient supply. The whole animal weights and body composition data were suggestive of adipose restriction, but this was not confirmed when individual adipose tissue weights were measured. The propensity to develop hepatomegaly with increased TG content early on, in the HF-fed NOD.B10 foz/foz mice, however, would be consistent with early “relative” adipose tissue restriction in this mouse group. Adipose dysfunction in HF-fed NOD.B10 foz/foz mice compared to Balb/c foz/foz mice was not evident from the adiponectin measurements. However, the high MCP-1 levels in HF-fed NOD.B10 foz/foz mice could be indicative of a greater propensity of this mice strain to develop adipose inflammation.

To further discern the status of the adipose tissue in the various mice groups, therefore, the mRNA expression of various adipose tissue-specific genes responsible for normal adipose physiology, adipokines and markers for inflammation were analyzed in the peri-ovarian adipose tissue.

3.6.1 Housekeeping genes

The mRNA expression levels of various markers of adipose tissue differentiation and function, adipokines and inflammation were evaluated at two different ages (6 and 12 weeks) for both strains in the peri-ovarian adipose tissue. Corrections for sample variations in the amount of starting peri-ovarian tissue sample, RNA integrity and cDNA sample amplified were carried out by the use of stable endogenous controls (housekeeping genes).
Expression of various housekeeping genes were tested, including: ribosomal protein L13a (RPL13a), β-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase-1 (HPRT-1), 18S rRNA, glyceraldehyde 3- phosphate dehydrogenase (GAPDH) and cyclophillin. Of these, RPL13a, B2M and HPRT-1 displayed significant differential expression between the two strains and were excluded from the study (Figure 27A-F). However, 18S rRNA, GAPDH and cyclophillin exhibited stable expression in both strains across all mice groups at 6 and 12 weeks of age (Figure 28A-F). Therefore, these three genes were chosen as housekeeping genes and the geometric mean of these housekeeping genes were used to normalize the mRNA expression of various genes during the analysis (Figure 29).
Figure 27: mRNA expression of housekeeping genes. (A-B) Ribosomal protein L13a (RPL13a) (A-B), (C-D) beta-2-microglobulin (B2M) and (E-F) hypoxanthine phosphoribosyltransferase-1 (HPRT-1) in WT and \textit{foz/foz} mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at 6 and 12 weeks of age. The expressions of these housekeeping genes were not stable across mice groups and were excluded from the experiment. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. $^{++}p<0.01$, $^{+++}p<0.001$, $^{++++}p<0.0001$, WT-chow vs foz-chow; $^#p<0.05$, $^{####}p<0.0001$, WT-HF vs foz-HF; $^*p<0.05$, $^{***}p<0.001$, NOD.B10 vs. Balb/c.
Figure 28: mRNA expression of stable housekeeping genes. (A-B) 18s rRNA, (C-D) glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and (E-F) cyclophillin in WT and $foz/foz$ mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at 6 and 12 weeks of age. The expressions of these genes were found to be stable across mice groups. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. No significant difference found.
Figure 29: Geometric mean of 3 housekeeping genes- 18s rRNA, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and cyclophillin expression in WT and foz/foz mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at (A) 6 weeks and (B) 12 weeks of age. This data was used to normalize the mRNA expression of various genes. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. No significant difference found.
3.6.2 mRNA expression of genes related to adipogenesis

Changes in adipose tissue differentiation in the NOD.B10 and Balb/c mice groups were determined by analysis of the mRNA expression of PPAR-γ, C/EBP-α and SREBP-1c, which are genes responsible for driving the final stage of adipogenesis and activating the transcription of various adipose-specific genes.

3.6.2.1 Peroxisome proliferator activated receptor-γ (PPAR-γ)

At 6 weeks of age, the mRNA expression of PPAR-γ was similar between mice groups within each strain (Figure 30A). Chow-fed Balb/c foz/foz mice displayed lower PPAR-γ expression compared to its NOD.B10 counterparts (0.6 ± 0.05 vs 1.0 ± 0.1, relative units, p<0.05).

At 12 weeks of age, there was no significant difference in the mRNA expression of PPAR-γ between NOD.B10 and Balb/c strains (Figure 30B). HF-feeding increased PPAR-γ expression in NOD.B10 WT mice (0.8 ± 0.1 vs 1.4 ± 0.1, relative units, WT-chow vs WT-HF, p<0.05) and Balb/c WT mice (0.6 ± 0.1 vs 1.0 ± 0.1, relative units, WT-chow vs WT-HF, p<0.05). HF-fed NOD.B10 foz/foz mice exhibited a non-significant trend for increased PPAR-γ expression at 12 weeks of age (Figure 30B).

3.6.2.2 CCAAT/enhancer-binding protein alpha (C/EBP-α)

The mRNA expression of C/EBP-α was similar in all the mice groups of both NOD.B10 and Balb/c strains at 6 and 12 weeks of age (Figure 30C-D).
3.6.2.3 Sterol regulatory element-binding protein-1c (SREBP-1c)

SREBP-1c also displayed similar expression in all the mice groups of both NOD.B10 and Balb/c strains at 6 and 12 weeks of age, apart from an increase with HF-feeding in NOD.B10 WT mice at 6 weeks of age only (Figure 30E-F).

Summary of results

The mRNA expression of PPAR-γ, C/EBP-α and SREBP-1c in response to HF-feeding, the foz/foz genotype and the combination of both, showed no consistent pattern in either mice strain at 6 or 12 weeks of age. As the expression of these genes did not decrease in the HF-fed foz/foz mice of either strain, there was no evidence of adipose dedifferentiation in these experiments.
Figure 30: mRNA expression of transcriptional factors and enzymes related to adipogenesis: (A-B) Peroxisome proliferator activated receptor-γ (PPAR-γ), (C-D) CCAAT/enhancer-binding protein-α (C/EBP-α) and (E-F) sterol regulatory element-binding protein-1c (SREBP-1c) in peri-ovarian WAT of WT and foz/foz mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at 6 and 12 weeks of age. PPAR-γ expression increased in HF-fed mice of both strains at 12 weeks of age, while SREBP-1c increased in HF-fed NOD.B10 WT mice at 6 weeks of age only. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, WT-chow v/s WT-HF; *p<0.05, NOD.B10 vs. Balb/c.
3.6.3 mRNA expression of genes related to fatty acid uptake and intracellular transport

Adipose tissue differentiation in the NOD.B10 and Balb/c foz/foz mice fed on HF diet was further determined by mRNA analysis of genes related to adipose tissue physiology, such as the uptake and intracellular transport of circulating fatty acids into the adipocytes for storage.

3.6.3.1 Lipoprotein lipase (LPL)

At 6 weeks of age, the mRNA expression of LPL was similar in all mice groups of both NOD.B10 and Balb/c strains (Figure 31A).

At 12 weeks of age, HF-fed NOD.B10 foz/foz mice displayed a significantly higher LPL expression than WT-HF mice (0.5 ± 0.05 vs 0.3 ± 0.03, relative units, respectively, p<0.01) and foz-chow mice (0.5 ± 0.07 vs 0.2 ± 0.04, relative units, respectively, p<0.001) (Figure 31B). However, no inter-strain difference in LPL expression was observed in any mice groups.

3.6.3.2 Angiopoietin-like-4 (ANGPTL4)

At 6 weeks of age, there was no change in ANGPTL4 expression between mice groups in both strains, other than higher ANGPTL4 expression in HF-fed NOD.B10 WT mice compared to its Balb/c counterparts (0.6 ± 0.08 vs 0.4 ± 0.06, relative units, p<0.01) (Figure 31C).

At 12 weeks of age, there was an increase in ANGPTL4 expression on HF-feeding of foz/foz mice of both strains (Figure 31D). HF-fed NOD.B10 foz/foz mice had almost double the expression of ANGPTL4 compared to chow-fed NOD.B10 foz/foz mice (1.0
± 0.07 vs 0.5 ± 0.05, relative units, respectively, \( p<0.0001 \) and WT-HF mice (1.0 ± 0.07 vs 0.5 ± 0.08, relative units, respectively, \( p<0.0001 \)). HF-fed Balb/c \( foz/foz \) mice also displayed similar increased ANGPTL4 expression compared to the foz-chow mice (0.6 ± 0.10 vs 0.3 ± 0.03, relative units, respectively, \( p<0.01 \)). However, ANGPTL4 expression in HF-fed Balb/c \( foz/foz \) mice was still lower compared to their NOD.B10 counterparts (0.6 ± 0.09 vs 1.0 ± 0.07, relative units, respectively, \( p<0.01 \)).

### 3.6.3.3 Stearoyl-CoA desaturase-1 (SCD-1)

At 6 and 12 weeks of age, HF-diet was found to drive the increase in mRNA expression of SCD-1 in both WT and \( foz/foz \) mice of NOD.B10 and Balb/c strains (Figure 31E-F). However, the response to HF diet was equivalent in both mice strains.

### 3.6.3.4 Fatty acid transporter protein- CD36

At 6 weeks of age, CD36 mRNA expression was similar in all mice groups of both strains (Figure 32A).

At 12 weeks of age, HF-fed NOD.B10 \( foz/foz \) mice displayed higher CD36 expression when compared to foz-chow mice (3.5 ± 0.4 vs 1.7 ± 0.1, relative units, \( p<0.001 \)) and WT-HF mice (3.5 ± 0.4 vs 1.3 ± 0.1, relative units, \( p<0.001 \)) (Figure 32B). HF-fed Balb/c \( foz/foz \) mice also displayed a similar increase in CD36 expression at 12 weeks of age when compared to foz-chow mice (2.1 ± 0.2 vs 1.7 ± 0.1, relative units, respectively, \( p<0.001 \)) and WT-HF mice (2.1 ± 0.2 vs 1.4 ± 0.2, relative units, respectively, \( p<0.001 \)).

However, no inter-strain differences in CD36 expression were discovered at both ages.
3.6.3.5 Adipose protein 2 (aP2)/Fatty acid binding protein 4 (FABP4)

At 6 weeks of age, similar mRNA expression of aP2/FABP4 was observed in all mice groups of both strains (Figure 32C).

However, at 12 weeks of age, chow-fed Balb/c WT mice exhibited higher aP2 expression compared to its NOD.B10 counterparts (0.7 ± 0.06 vs 0.4 ± 0.06, relative units, respectively, p<0.05) (Figure 32D). HF-fed NOD.B10 WT mice had increased aP2 expression compared to WT-chow mice (0.7 ± 0.05 vs 0.4 ± 0.06, relative units, respectively, p<0.05).

Summary of results

HF diet induced the expression of SCD-1 which is an important enzyme of the fatty acid esterification process. This effect of HF diet was not altered by the presence of the foz/foz genotype or differentially between mice strains. The combination of HF diet and the foz/foz genotype caused increased expression of CD36 at 12 weeks of age, which is involved in fatty acid transport. Again, mice strain did not affect the adipose tissue CD36 response. Expression of the LDL inhibitor ANGPTL4, however, had increased expression in the NOD.B10 mice compared to the Balb/c mice at 6 weeks of age when WT mice were fed HF diet, and at 12 weeks of age when foz/foz mice were fed HF diet. Overall, though, the effects of HF diet and the foz/foz genotype to affect this panel of fatty acid uptake and intra-cellular partitioning genes differed little between the two mice strains.
Figure 31: mRNA expression of genes related to fatty acid uptake and intracellular transport in periovarian WAT: (A-B) Lipoprotein lipase (LPL), (C-D) angiopoietin like-4 (ANGPTL4) and (E-F) stearoyl-CoA desaturase-1 (SCD-1) in WT and foz/foz mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at 6 and 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, §§p<0.01, §§§p<0.001, WT-chow vs WT-HF; +++p<0.001, foz-chow vs WT-chow; ♯♯p<0.01, ♯♯♯♯p<0.0001, WT-HF vs foz-HF, ***p<0.001, ****p<0.0001 foz-chow vs foz-HF; **p<0.01, NOD.B10 vs Balb/c.
Figure 32: mRNA expression of genes related to fatty acid uptake and intracellular transport in periovarian WAT: (A-B) Fatty acid transporter protein CD36 and (C-D) adipose protein 2 (aP2) in WT and foz/foz mice of NOD.B10 and Balb/c strains fed on chow and HF diet, at 6 and 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, WT-chow vs WT-HF; §§§§p<0.0001, WT-HF vs foz-HF; §§§p<0.001, foz-chow vs foz-HF; *p<0.05, NOD.B10 vs Balb/c.
3.6.4 mRNA expression of genes related to lipolysis and lipid droplet formation

The mRNA expression of genes responsible for driving lipolysis and lipid droplet formation in adipocytes were analyzed to determine any changes in adipose tissue function between the various groups of the two mouse strains at 6 and 12 weeks of age.

3.6.4.1 G0/G1 switch gene 2 (G0S2)

At 6 weeks of age, G0S2 expression was unaltered in all mice groups of both strains (Figure 33A).

At 12 weeks of age, HF-fed foz/foz mice of both strains exhibited a non-significant trend for reduced G0S2 mRNA expression compared to their WT controls (Figure 33B). However, no inter-strain differences in G0S2 mRNA expression were observed at both ages.

3.6.4.2 Adipose triglyceride lipase (ATGL)

At 6 weeks of age, ATGL mRNA expression was observed to be reduced in the HF-fed NOD.B10 foz/foz mice compared to the WT-HF controls (0.4 ± 0.04 vs 0.9 ± 0.09, relative units, respectively, p<0.001), as well as in HF-fed Balb/c foz/foz mice compared to WT-HF mice (0.5 ± 0.04 vs 0.8 ± 0.07, relative units, respectively, p<0.001) (Figure 33C).

At 12 weeks of age, the down-regulation of ATGL expression was also prevalent in HF-fed NOD.B10 foz/foz mice compared to WT-HF mice (0.5 ± 0.06 v/s 0.7 ± 0.03, relative units, respectively, p<0.01), as well as in HF-fed Balb/c foz/foz mice compared to WT-HF mice (0.4 ± 0.04 v/s 0.7 ± 0.06, relative units, respectively, p<0.01) (Figure 33D).
3.6.4.3 Hormone sensitive lipase (HSL)

At 6 weeks of age, the mRNA expression of the lipolytic enzyme HSL displayed a similar pattern to ATGL expression, where HF-feeding caused reduction in expression in *foz/foz* mice of both strains (Figure 33E).

At 12 weeks of age, interestingly, HF-feeding led to an up-regulation of HSL expression in the NOD.B10 and Balb/c WT mice compared to WT-chow controls (Figure 33F). Conversely, HF-fed NOD.B10 *foz/foz* mice exhibited reduced HSL expression when compared to WT-HF mice (0.6 ± 0.03 vs 0.9 ± 0.09, relative units, respectively, p<0.001). However, no significant inter-strain difference in HSL expression was observed in any mice groups of both strains (Figure 33E-F).

3.6.4.4 Adipose differentiation related protein (ADRP)

At 6 weeks of age, mRNA expression of ADRP was similar between mice groups within each strain. However, inter-strain differences in mRNA expression were observed in the chow-fed Balb/c WT mice, which had higher ADRP expression compared to its NOD.B10 counterpart (0.8 ± 0.04 vs 0.4 ± 0.05, relative units, respectively, p<0.01) (Figure 33G).

At 12 weeks of age, a slight tendency for increased ADRP expression was evident in HF-fed WT mice of both strains. Furthermore, HF-fed NOD.B10 *foz/foz* mice displayed a significant elevation in ADRP mRNA expression compared to the foz-chow mice (1.7
± 0.10 vs 0.6 ± 0.05, relative units, respectively, p<0.0001) and WT-HF mice (1.7 ± 0.10 vs 0.6 ± 0.06, relative units, respectively, p<0.0001) (Figure 33H). Similarly, HF-fed Balb/c foz/foz mice had higher ADRP expression than the foz-chow mice (1.6 ± 0.10 vs 0.7 ± 0.04, relative units, respectively, p<0.0001) and WT-HF mice (1.6 ± 0.10 vs 0.9 ± 0.10, relative units, respectively, p<0.0001). However there were no significant inter-strain differences in the mRNA expression of ADRP in any mice group.

Summary of results

ATGL and HSL, which are both lipolytic enzymes responsible for the breakdown of TG, exhibited reduced expression at the mRNA level in the HF-fed foz/foz mice of both strains, compared to WT controls, suggesting down-regulation of lipolysis in the periovarian adipocytes of these mice. This is at variance from the effect of HF-feeding in WT mice in which HSL mRNA expression increased in both strains. ADRP, which is involved in the formation of lipid droplets in adipocytes, had higher expression in the HF-fed foz/foz mice of both strains. The net effect of reduced lipolytic activity with increased lipid droplet associated protein, would favor the accumulation of more TG in the adipose tissue of HF-fed foz/foz mice that have the greatest excess nutrient load to accommodate. However, there was no obvious difference between the NOD.B10 and Balb/c mice strains in this adipose tissue response.
Figure 33: mRNA expression of genes related to lipolysis and lipid droplet inflammation in peri-ovarian WAT: (A-B) G0/G1 switch gene 2 (G0S2), (C-D) adipose triglyceride lipase (ATGL) and (E-F) hormone sensitive lipase and (G-H) adipose differentiation related protein (ADRP) in WT and foz/foz mice of NOD.B10 and Balb/c strains fed on chow or HF diet at 6 and 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §§§p<0.001, WT-chow vs WT-HF; ♯♯♯p<0.01, ♯♯♯♯p<0.001, ♯♯♯♯♯p<0.0001, WT-HF vs foz-HF; ****p<0.0001, foz-chow vs foz-HF; **p<0.01, NOD.B10 vs Balb/c.
3.6.5 mRNA expression of adipokines

Adipose tissue dysfunction leads to altered adipokine profiles. Therefore, the effects of excess nutrition due to HF-feeding and the foz/foz genotype were determined by analyzing the mRNA expression of various adipokines at 6 and 12 weeks of age in the peri-ovarian adipose tissue of both strains.

3.6.5.1 Adiponectin

At 6 weeks of age, the mRNA expression of adiponectin was similar in most mice groups in both strains, with the exception of HF-fed NOD.B10 foz/foz mice which displayed reduced adiponectin expression compared to the WT-HF mice (0.5 ± 0.1 vs 0.8 ± 0.1, relative units, p<0.05) (Figure 34A).

At 12 weeks of age, HF-fed NOD.B10 foz/foz mice developed a reduction in adiponectin mRNA levels compared to the foz-chow mice (0.2 ± 0.04 vs 0.7 ± 0.05, relative units, p<0.001) and WT-HF mice (0.2 ± 0.04 vs 0.8 ± 0.07, relative units, p<0.001) (Figure 34B). Similarly, HF-fed Balb/c foz/foz mice had reduced adiponectin expression when compared to the WT-HF mice (0.4 ± 0.03 vs 0.8 ± 0.15, relative units, p<0.001). No inter-strain differences in adiponectin expression were observed in any mice groups.

3.6.5.2 Leptin

At 6 weeks of age, the mRNA expression of leptin trended higher, although not significantly, with HF-feeding in WT and foz/foz mice of both strains (Figure 34C). Leptin levels were generally higher in the NOD.B10 mice compared to Balb/c mice, but
this was only significant for HF-fed WT mice (0.8 ± 0.2 vs 0.4 ± 0.1, relative units, WT-HF NOD.B10 vs Balb/c, p<0.001).

At 12 weeks of age, HF-feeding caused an increase in leptin expression in the WT mice of both strains (Figure 34C). The foz/foz genotype also caused increased leptin expression in chow-fed NOD.B10 foz/foz mice, which displayed an 8.5 fold increase in leptin expression compared to WT-chow mice (1.7 ± 0.1 vs 0.2 ± 0.1, relative units, p<0.001). The combination of HF-feeding and the foz/foz genotype led to 3.6 times up-regulation of leptin expression in the NOD.B10 foz/foz mice when compared to WT-HF mice (2.2 ± 0.1 vs 0.6 ± 0.1, relative units, p<0.001). Balb/c foz/foz mice on chow and HF diet also displayed a similar pattern.

However, inter-strain differences in mRNA expression were observed where leptin expression was almost 30% higher in chow-fed NOD.B10 foz/foz mice compared to their Balb/c counterparts (2.2 ± 0.1 vs 1.5 ± 0.1, relative units, p<0.01) as well as HF-fed NOD.B10 foz/foz mice compared to their Balb/c counterparts (1.7 ± 0.1 vs 1.0 ± 0.1, relative units, p<0.001).

3.6.5.3 Tumor necrosis factor-α (TNF-α)

At 6 weeks of age, the mRNA expression of TNF-α was not altered in any mice groups in both strains (Figure 34E).

However, at 12 weeks of age, HF-fed NOD.B10 foz/foz mice developed significantly elevated TNF-α mRNA expression compared to WT-HF mice (0.2 ± 0.07 vs 0.08 ± 0.03, relative units, p<0.01)(Figure 34F). Nonetheless, there was no difference in TNF-α expression between NOD.B10 and Balb/c strains at both 6 and 12 weeks of age.
3.6.5.4 Interleukin-6 (IL-6)

At 6 weeks of age, the mRNA expression of IL-6 was similar in all mice groups within each strain, except chow-fed NOD.B10 foz/foz mice which exhibited higher IL-6 expression compared to its Balb/c counterpart (0.9 ± 0.1 vs 0.4 ± 0.1, relative units, p<0.05) (Figure 34G).

At 12 weeks of age, HF-fed NOD.B10 foz/foz mice had almost double the expression of IL-6 when compared to foz-chow mice (1.8 ± 0.2 vs 0.8 ± 0.1, relative units, respectively, p<0.001) and WT-HF mice (1.8 ± 0.2 vs 0.7 ± 0.1, relative units, respectively, p<0.0001) (Figure 34H). HF-fed Balb/c foz/foz mice also displayed a similar pattern of IL-6 mRNA expression. However, there were no inter-strain differences in IL-6 expression.

Summary of results

As expected with obesity, and in agreement with plasma levels, the mRNA expression of adiponectin was reduced in the HF-fed foz/foz mice of both strains. Leptin expression was increased in the foz/foz mice of both strains, with higher expression in the NOD.B10 mice. The expression of pro-inflammatory cytokines TNF-α and IL-6 was observed to be increased in the HF-fed foz/foz mice of both strains suggesting the presence of inflammation in these mice by 12 weeks of age, but not at 6 weeks of age. Overall, these results are indicative of the presence of adipose tissue dysfunction and inflammation in response to HF-feeding and the foz/foz genotype by 12 weeks of age, in both strains of mice. The findings that leptin mRNA levels were higher in NOD.B10 mice are of interest, but alone do not suggest a greater degree of dysfunction in response to overfeeding in this strain compared to Balb/c mice.
Figure 34: mRNA expression of adipokines: (A-B) adiponectin, (C-D) leptin, (E-F) tumour necrosis factor-α (TNF-α) and (G-H) interleukin-6 (IL-6) in NOD.B10 and Balb/c mice in peri-ovarian WAT. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. ++++p<0.0001, WT-chow vs foz-chow; ♯p<0.01, ♯♯p<0.05, ♯♯♯p<0.001, ♯♯♯♯p<0.0001, WT-HF vs foz-HF; ¶¶p<0.01, ¶¶¶p<0.001, ¶¶¶¶p<0.0001, foz-chow vs foz-HF; *p<0.05, **p<0.01, ***p<0.001, NOD.B10 vs Balb/c.
3.6.6 mRNA expression of genes related to inflammation

To assess the effects of HF-feeding and the foz/foz genotype on adipose inflammation in the two mice strains, the mRNA expression of markers for inflammation was analyzed.

3.6.6.1 Cluster of differentiation 68 (CD68)

At 6 weeks of age, the mRNA expression of CD68 was not altered in any of the mice groups in both strains (Figure 35A).

At 12 weeks of age, CD68 expression in HF-fed NOD.B10 foz/foz mice was highly increased when compared to foz-chow mice (7.1 ± 0.6 vs 1.7 ± 0.4, relative units, p<0.0001) and WT-HF mice (1.7 ± 0.4 vs 0.8 ± 0.2, relative units, p<0.0001) (Figure 35B). HF-fed Balb/c foz/foz mice also displayed a similar up-regulation in CD68 expression compared to its controls. However, CD68 expression in HF-fed NOD.B10 foz/foz mice was more prominent compared to its Balb/c counterpart (7.1 ± 0.6 vs 3.1 ± 0.6, relative units, p<0.0001).

3.6.6.2 Cluster of differentiation 11c (CD11c)

At 6 weeks of age, the mRNA expression of CD11c was similar between all mice groups in both strains (Figure 35C).

At 12 weeks of age, the mRNA expression of CD11c was found to be highly increased in HF-fed NOD.B10 foz/foz mice when compared to foz-chow mice (3.6 ± 0.3 vs 1.1 ± 0.1, relative units, p<0.0001) and WT-HF mice (3.6 ± 0.3 vs 0.3 ± 0.1, relative units, p<0.0001) (Figure 35D). The same pattern was also observed in the HF-fed Balb/c
foz/foz mice. There was no significant inter-strain difference in CD11c mRNA expression in any of the mice groups.

3.6.6.3 Monocyte chemoattractant protein-1 (MCP-1)

At 6 weeks of age, no significant difference in the mRNA expression of MCP-1 was observed between the mice groups of both strains (Figure 35E). However, inter-strain differences in MCP-1 expression were observed at this age. HF-fed NOD.B10 WT mice displayed higher MCP-1 expression compared to its Balb/c counterparts (0.05 ± 0.01 vs 0.02 ± 0.003, relative units, p<0.05). Chow-fed NOD.B10 foz/foz mice also showed higher MCP-1 expression compared to their Balb/c counterparts (0.04 ± 0.01 vs 0.02 ± 0.003, relative units, p<0.05) (Figure 35E).

At 12 weeks of age, Balb/c WT mice increased MCP-1 expression only with HF-feeding (Figure 35F). However, a major up-regulation in MCP-1 expression was noticeable in the chow-fed NOD.B10 foz/foz mice compared to WT-chow mice (0.3 ± 0.1 vs 0.04 ± 0.01, relative units, p<0.0001). The combination of HF-diet and the foz/foz genotype in NOD.B10 foz/foz mice further increased MCP-1 expression at 12 weeks of age, when compared to WT-HF mice (0.6 ± 0.04 vs 0.06 ± 0.02, relative units, p<0.0001). However, HF-fed Balb/c foz/foz mice did not exhibit similar up-regulation of MCP-1 expression at this age.

Inter-strain differences in MCP-1 expression was observed with the up-regulation of MCP-1 mRNA found to be significantly higher in the chow-fed NOD.B10 foz/foz mice compared to their Balb/c counterparts (0.35 ± 0.06 vs 0.07 ± 0.001, relative units, p<0.0001), as well as in the HF-fed NOD.B10 foz/foz mice compared to their Balb/c counterparts (0.63 ± 0.04 vs 0.12 ± 0.03, relative units, p<0.0001).
Summary of results

The combination of HF-feeding and the *foz/foz* genotype clearly causes inflammation in both mice strains; however, the pattern is not the same between strains. The CD68 response, a marker of macrophage infiltration, was greater in the HF-fed *foz/foz* mice of NOD.B10 strain compared to the Balb/c mice at 12 weeks of age. More remarkably different between strains was the MCP-1 response, which was dramatically increased in both chow-fed and HF-fed NOD.B10 *foz/foz* mice compared to Balb/c mice which had no increase in MCP-1 mRNA levels. These results suggest the findings of the higher circulating MCP-1 levels found in the HF-fed NOD.B10 *foz/foz* mice compared to their Balb/c counterparts at 12 weeks of age being at least partly from adipose tissue. Also of note in these results is the complete absence of these inflammatory markers at 6 weeks of age, suggesting these changes occur later than this age.
Figure 35: mRNA expression of genes related to inflammation in periovarian WAT: (A-B) CD68, (C-D) CD11c and (E-F) monocyte chemoattractant protein-1 (MCP-1) in NOD.B10 and Balb/c mice strains at 6 and 12 weeks of age. Data are means ± SEM of 10 mice per group. Two-way ANOVA with Bonferroni post-hoc test. §p<0.05, WT-chow vs WT-HF; ++++p<0.0001, WT-chow vs foz-chow; iciónp<0.0001, WT-HF vs foz-HF; igatorp<0.0001, foz-chow vs foz-HF; *p<0.05, ****p<0.0001, NOD.B10 vs Balb/c.
4. Discussion and Conclusion

4.1 Discussion and Conclusion

The rapid increase in global obesity rates have led to a massive health and economic burden with increased mortality arising from obesity-related co-morbidities such as T2D and NASH.\cite{6, 48, 83} To better understand the determinants for the development of T2D and NASH in chronically over-nourished individuals may lead to new modalities of therapy for disease prevention. This thesis has focussed on the potential role of a dysfunctional response of adipose tissue to over-nutrition, T2D and NASH pathogenesis using \textit{Alms 1} mutant mice on different backgrounds. Past studies by Larter \textit{et al} on the female NOD.B10 \textit{foz/foz} mice demonstrated that after 24 weeks on HF diet, these mice develop obesity, adipose restriction, liver injury, inflammation and resultant hepatic lipid overload resulting in NASH.\cite{360} Preliminary studies on the Balb/c \textit{foz/foz} mice on the same HF diet displayed only the development of obesity, with no associated metabolic conditions, suggesting that these mice are diabetes-resistant and do not develop NASH. The dramatic strain differences in these mice models therefore allow us to characterize the underlying metabolic interactions between adipocytes, islet β-cells and hepatocytes in response to HF feeding. Thus, the overall aim was to determine the role of adipose tissue in the determination of a healthy obesity response or obesity associated with metabolic diseases in Balb/c mice compared to NOD.B10 mice.

The first specific aim was to determine the early response and time-course of adipose tissue expansion, gene expression and inflammation, together with a secreted adipokine profile, of NOD.B10 and Balb/c mice, to the overfeeding challenges of HF diet, the \textit{foz/foz} genotype and the combination of both.
As predicted, the combination of HF feeding and the foz/foz genotype promoted marked obesity in both strains of mice, with the suggestion from whole body weight measurements and DEXA, that initial weight gain up to 8 weeks of age (or 4 weeks on HF diet) was greater in the HF-fed NOD.B10 foz/foz mice than their Balb/c counterparts. Furthermore, the adipose tissue expansion in HF-fed NOD.B10 foz/foz mice, but not Balb/c mice, appeared to slow from 10 to 12 weeks of age suggesting the possibility of adipose restriction commencing at this time in the NOD.B10 strain. The finding that the livers of the HF-fed NOD.B10 foz/foz mice were accumulating more TG than the HF-fed Balb/c foz/foz mice would also be consistent with this, as this suggests overflow of fatty acid storage from adipose tissue to the liver. However, when the weights of individual fat depots were measured, adipose tissue restriction could not be found. A possible explanation for this discrepancy is that only select adipose depots rather than the total depots were being sampled.

These differences in initiation of weight gain could be attributed to variations in food intake and energy expenditure, arising from strain differences, which will be a focus for future investigations.

By 12 weeks of age, the effects of HF feeding and the foz/foz genotype on adipose tissue differentiation factors were minimal with no obvious strain differences. With respect to genes relating to adipocyte function, the combination of HF feeding and the foz/foz genotype certainly caused changes by 12 weeks of age. For example, SCD-1, CD36, ANGPTL4, and ADRP were all increased in HF-fed foz/foz mice of both strains, although the effect was significantly greater for ANGPTL4 in the NOD.B10 compared to the Balb/c strain. Similarly, the lipolysis genes ATGL and HSL were similarly reduced in HF-fed foz/foz compared to HF-fed WT mice in both strains of mice. Thus,
these functional changes appear to be adaptation to the marked excess nutrient load that is unrelated to whether the mice develop T2D or NASH.

The mRNA expression of the adipose tissue inflammatory cytokines TNF-\(\alpha\) and IL-6 were increased and the expression of the adipokine adiponectin was decreased in the peri-ovarian adipose tissue of HF-fed \(foz/foz\) mice at 12 weeks of age similarly in both strains of mice, indicative of MetS-like adipose tissue changes induced by the excess nutrient supply in both strains. Adiponectin levels inversely correlate to obesity and can predict severity of steatosis and transition to NASH, with positive association to insulin sensitivity.\(^{[316]}\) The expression of leptin was also increased in HF-fed \(foz/foz\) mice of both strains, with a greater effect in the NOD.B10 mice. Although the significance of this is unclear, leptin is known to increase the expression of pro-inflammatory adipokines like TNF-\(\alpha\), IL-6 and MCP-1.\(^{[316]}\).

Obesity is associated with macrophage infiltration and increased expression of pro-inflammatory adipocytokines.\(^{[362]}\) Of particular interest was the finding of much greater increases of CD68, a marker for M1 macrophages, and MCP-1 by 12 weeks of age in HF-fed \(foz/foz\) mice of NOD.B10 strain compared to Balb/c mice, suggestive of greater inflammation in the adipose tissue of the over-nourished NOD.B10 mice. MCP-1 expression increases in obese mice and its expression promotes monocyte infiltration, which differentiate into adipose tissue macrophages (ATM) further aggravating inflammation by secreting cytokines.\(^{[363]}\) These findings were not present at 6 weeks of age. Unfortunately, data is not available to indicate at what age these changes commenced in the HF-fed NOD.B10 \(foz/foz\) mice.

In keeping with the adipose mRNA levels, plasma adiponectin levels were mildly reduced and plasma MCP-1 levels were increased in HF-fed \(foz/foz\) mice of both mouse
strains. Furthermore, MCP-1 levels were higher in HF-fed NOD.B10 *foz/foz* mice compared to their Balb/c counterparts.

The second specific aim was to determine the temporal relationship between the adipose tissue responses to the overfeeding challenges in the two mice strains and the development of T2D and NASH.

Despite similar development of obesity in both NOD.B10 and Balb/c *foz/foz* mice on HF diet, only the HF-fed NOD.B10 *foz/foz* mice exhibited early evidence of impaired glucose tolerance at 5 weeks of age and developed hyperglycaemia at 8 weeks of age, which progressed to diabetes by 12 weeks of age. Interestingly, this was associated with profound hyperinsulinaemia evident at 8 weeks of age, indicating that despite increased plasma insulin levels, normal post-prandial glucose levels could not be maintained in these mice. On the other hand, HF-fed Balb/c *foz/foz* mice maintained normoglycaemia with moderate elevation of plasma insulin levels. The severe hyperinsulinaemia in the NOD.B10 mice could be suggestive of very severe insulin resistance developing in these mice, defective insulin clearance, possibly the presence of circulating insulin-specific antibodies binding to and inactivating the insulin molecules, or abnormal processing of insulin producing less active insulin demanding huge compensatory responses from the β-cells of the NOD.B10 strain. These abnormalities in glucose regulation and insulin secretion are very early compared to the changes that develop in adipose tissue.

Similar to observations made in long-term studies, NOD.B10 *foz/foz* mice developed hepatomegaly, hepatic injury and transition from simple steatosis to NASH when fed on HF diet, whereas the Balb/c mice did not. Hepatomegaly development was already evident at 8 weeks of age, which was earlier than expected. Although increased hepatic
TG content, expressed as mg/g, developed similarly in both mice strains, HF-fed NOD.B10 foz/foz mice had a greater total hepatic TG content, suggesting that the hepatomegaly observed in these mice could be due to abnormal ectopic TG deposition. ALT was used as a marker of hepatocellular damage. ALT levels increased in the HF-fed NOD.B10 foz/foz mice only and this occurred from 10 weeks of age after the development of hyperglycaemia and hyperinsulinaemia.

Despite development of obesity, T2D and NASH in NOD.B10 foz/foz mice fed on HF diet by 12 weeks of age, the evidence from our temporal studies suggested no major effect on adipose tissue restriction within the time frame of the study. Adipose restriction may occur to a greater degree later on, since previous studies have shown that the onset of adipose restriction in NOD.B10 foz/foz mice occurs after 10 weeks on HF diet, whereas this study ended at 12 weeks of age (8 weeks on HF diet).

In summary, these studies demonstrate that by 12 weeks of age, HF-fed NOD.B10 foz/foz mice already developed obesity and T2D, in parallel with development of hepatomegaly, hepatocellular injury, increased hepatic TG content and NASH, without strong evidence for concomitant adipose restriction or adipose dedifferentiation; whereas the Balb/c foz/foz mice remained diabetes and NASH resistant. A pro-inflammatory environment occurs in both mice strains, leading to chronic low grade inflammation associated with obesity and obesity-induced insulin resistance. Hyperglycaemia and hyperinsulinaemia clearly differentiated the NOD.B10 metabolic disease prone mice from the Balb/c metabolic disease resistant strain and both these anomalies occurred very early on. Therefore, it can be inferred that adipose tissue plays a secondary role in the pathogenesis of the metabolic disorders in this mouse model, while islet β-cell dysfunction, including the quality of the insulin produced, may be the primary factor responsible for the development of both adipose tissue dysfunction and
NAFLD and the triggering of the progression to NASH. These results do not preclude positive pathogenic feedback loops between the islet $\beta$-cell, adipocytes and hepatocytes in the progressive worsening in the metabolic state of HF-fed NOD.B10 $foz/foz$ mice.

### 4.2 Limitations of study

#### 4.2.1 Monitoring at different ages

NOD.B10 mice were monitored at 6, 8, 10 and 12 weeks of age, whereas Balb/c mice were only monitored at 6 and 12 weeks of age. Since Balb/c mice were the control mice, no major changes were expected before 12 weeks of age. However, as Balb/c $foz/foz$ mice on HF diet displayed a different pattern in the development of obesity, it would be worthwhile to conduct studies on the Balb/c mice at 8 and 10 weeks of age, similar to the NOD.B10 mice.

The mRNA expression of adipose-specific genes, adipokines and inflammatory markers were only analysed at 6 and 12 weeks of age. Since no adipose dedifferentiation or loss of function was observed in both mice strains at 12 weeks of age, analysis of mRNA expression before this age is not required. However, the results show onset of hyperglycaemia and hepatomegaly at 8 weeks in the NOD.B10 $foz/foz$ mice and NASH appears earlier than 12 weeks of age. The mRNA expression of inflammatory markers was also highly increased in both mice strains at 12 weeks of age. Therefore it may be advisable to analyse the expression of these markers at an earlier age to detect age of onset of inflammation in these mice.
4.2.2 Expression of genes at mRNA level only

The expression of adipogenic genes and adipokines were studied by measuring their mRNA levels. However, various studies that demonstrated that the mRNA levels of a gene do not always correlate to its protein levels, due to factors such as translational and post-translational modifications, RNA secondary structures and protein half-lives.\[364\] Therefore measurement of both mRNA and protein levels is recommended to determine gene expression. Since in this study, circulating plasma protein levels of all the genes were not analysed, Western Blot or sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled with mass spectrometry would substantiate the results obtained from the mRNA levels of these genes.

4.3 Proposed subsequent investigations

4.3.1 Food intake studies

Balb/c \textit{foz/foz} mice fed on HF diet displayed later onset of obesity at 8 weeks of age, compared to its NOD.B10 counterparts. Therefore, measurement of food intake of these mice could possibly determine whether differences in food consumption caused a different pattern in weight gain in these mice. Preliminary experiments on the food intake of the Balb/c mice have already been initiated, with food consumption measurements taken over 3 days in each week from 4 to 12 weeks of age, along with body weight measurements. Balb/c mice fed on HF diet displayed higher calorie intake compared to the chow-fed mice. However, food intake measurements of NOD.B10 mice, conducted currently with Balb/c mice, are also necessary to determine any variations in calorie intake, and will be conducted during future investigations.
4.3.2 Gene expression in subcutaneous adipose tissue

In this study, only peri-ovarian adipose tissue was used to determine the mRNA expression of various adipogenic genes and adipokines, and inflammatory markers such as CD68 and CD11c. However, studies have shown that the expression of certain genes and adipokines are different in the adipose tissue depending on their location. Visceral adipose tissue is associated with hypoadiponectinemia and increased IL-6 secretion. Therefore, analysis of the mRNA expression of these genes in more subcutaneous adipose and visceral (e.g. mesenteric) tissue sites could be conducted to corroborate whether adipose dedifferentiation or inflammation occurs in both these adipose tissue depots at 12 weeks of age.

4.3.3 Qualitative studies

In this study, adipose restriction and dysfunction was identified through quantitative experiments such as mRNA expression of adipogenic genes and plasma levels of adipokines. Similarly, the presence of inflammation was predicted from increased mRNA expression of inflammatory markers. In the liver, steatosis and progression of NAFLD to NASH was deduced from increased hepatic TG content and elevated plasma ALT levels.

Therefore, qualitative experiments such as Oil Red-O staining to illustrate hepatic steatosis, and immunohistochemistry (IHC) analysis of adipose tissue and liver to outline the presence of inflammatory macrophages and crown-like structures, could provide visual corroboration to results obtained quantitatively in this study.
4.3.4 Use of knock out mice

The results of this study suggest that the adipose tissue inflammatory changes, in particular the increase in MCP-1, in the HF-fed NOD.B10 flox/flox mice occurs after the development of hyperglycaemia and hyperinsulinaemia. In order to more convincingly show the hyperglycaemia and hyperinsulinaemia develop independently of adipose tissue inflammation, the MCP-1 knock out mice could be backcrossed on to NOD.B10 WT Alms 1 mutant mice. As MCP-1 is expressed in multiple tissues, an MCP-1 adipose specific MCP-1 knock out mouse would be of particular interest.
Solutions:

The constituents and preparation of solutions mentioned in Chapter 2 are shown below.

**DNA Lysis buffer**

For preparation DNA lysis buffer, the following solutions were constituted:

- 1 M Tris pH 8.0: Added 60.57 g of Trizma® base (Cat# T1503, Sigma Aldrich, CA, USA) in 500 mL of Milli-Q water and pH adjusted to 8.0 using 10 mol/L NaOH (Cat# S0899, Sigma Aldrich, CA, USA) and pH meter (Orion STAR A211, Thermo Scientific, MA, USA)
- 0.1 M EDTA: Added 7.44 g of EDTA (Cat# E9884, Ethylene diaminetetraacetic acid, Sigma Aldrich, CA, USA) in 200 mL of Milli-Q water
- 5 M NaCl: Added 58.44 g of NaCl (Cat# S5886, Sigma Aldrich, CA, USA) in 200 mL of Milli-Q water
- 10% SDS: Added 20 g Sodium Dodecyl sulfate (Cat# L4390, Sigma Aldrich, CA, USA) in 200 mL of Milli-Q water

For preparation of 1 L DNA lysis buffer, the following were added:

- 100 mL of 1 M Tris pH 8.0
- 50 mL of 0.1 M EDTA
- 40 mL of 5 M NaCl
- 20 mL of 10% SDS

The solution was topped up to 1 L with Milli-Q water, then sterile-filtered using 0.22 μm filter (Millipore Corporation, Massachusetts, USA) and stored at room temperature.
**70% ethanol**

For preparation of 70% ethanol, the following solutions were constituted:

- 70 mL absolute ethanol (cat# 214, Univar, Ajax FineChem, NSW, Australia)
- 30 mL Milli-Q water

**75% ethanol**

For preparation of 75% ethanol, the following solutions were constituted:

- 75 mL absolute ethanol (cat# 214, Univar, Ajax Fine Chem, NSW, Australia)
- 25 mL Milli-Q water

**TE Buffer**

For preparation of 1 L of TE buffer, the following solutions were constituted:

- 10 mM Tris-HCl pH 8.0 (Triethanolamine hydrochloride, Cat# T1502, Sigma Aldrich, CA, USA)
- 0.5 M EDTA: (Cat# E9884, Sigma Aldrich, CA, USA)

For preparation of 1 L of TE buffer, the following were added:

- 1.21 g of 10 mM Tris-HCl pH 8.0
- 2 mL of 0.5 M EDTA

The solution was pH adjusted to 8.0 using NaOH and topped up to 1 L with Milli-Q water, sterile-filtered using 0.22 µm filter (Millipore Corporation, Massachusetts, USA) and stored at room temperature.
**TAE buffer (Tris-Acetic acid-ethylenediaminetetracetic acid buffer)**

For preparation of 1 L of 50X TAE buffer, the following were added:

- 242 g of Tris (Trizma® base, Cat# T1503, Sigma Aldrich, CA, USA)
- 18.61 g of EDTA (Ethylenediaminetetra acetic acid, Cat# E9884, Sigma Aldrich, CA, USA)
- 57.1 mL of glacial acetic acid (Cat# 537020, Sigma Aldrich, CA, USA)

The solution was topped up to 1 L with Milli-Q water, then sterile-filtered using 0.22 µm filter (Millipore Corporation, Massachusetts, USA) and stored at room temperature.

A running solution of 1X TAE buffer was then prepared by diluting 10 mL of 50X TAE buffer in 990 mL of Milli-Q water.

**KOH (Potassium Hydroxide)**

For preparation of 0.5 M KOH, the following were added:

- 5.6 g KOH (Cat# P5958, Sigma, CA, USA)
- 200 mL of 95% ethanol (prepared by diluting 190 mL absolute ethanol (Cat# 214, Univar, Ajax Fine Chem, NSW, Australia) in 10 mL of Milli-Q water)

**MgSO₄ (Magnesium Sulfate)**

For the preparation of 0.15 M MgSO₄, the following were added:

- 11.07 g of MgSO₄.7H₂O (Cat# 230391, Sigma Aldrich, CA, USA)
- 300 mL of Milli-Q water
DEPC-water

For preparation of 1 L of DEPC-water, the following were added:

- 1 mL of diethyl pyrocarbonate (Cat # D5758, Sigma Aldrich, CA, USA)
- 1 L of Milli-Q water

The solution was stirred overnight with a magnetic stirrer at 37°C and autoclaved before use.

Loading buffer for electrophoresis

For the preparation of loading buffer, the following were added:

- 25 mg of bromophenol blue (Cat# B8016, Sigma, CA, USA)
- 4 g of sucrose (Cat# S0389, Sigma, CA, USA)
- 10 mL of Milli-Q water

Figure 37: Mice ear notch chart for identification of each mouse.
Bibliography:


76. Registeries, N., AloHaWaAAoC. 2006.


81. Speliotes, E.K., et al., Genome-wide association analysis identifies variants associated with nonalcoholic fatty liver disease that have distinct effects on metabolic traits. PLoS Genet, 2011. 7(3): p. e1001324.


