PREDISPOSITION TO NON ALCOHOLIC FATTY LIVER DISEASE IN PRETERM GUINEA PIGS.

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Abstract

**Background**: Preterm birth accounts for ~10% of all births and is now an established risk factor for the development of metabolic syndrome, a cluster of conditions responsible for significant morbidity and mortality in New Zealand. The mechanisms underlying the development of Non alcoholic fatty liver disease (NAFLD, the hepatic manifestation of metabolic syndrome) in the ex-preterm are unknown at present. This project investigated potential differences between term and preterm born guinea pigs in the biological pathways believed to be associated with non-alcoholic fatty liver disease, including metabolism, and molecular and physiologic changes within the liver.

**Methods**: Guinea pigs were delivered spontaneously at term (~GA69days), or prematurely by pharmacological induction of labour (GA62; equivalent to 32 weeks in humans). Fasting blood sugar levels (BSL) were taken at birth, and weekly thereafter. At corrected postnatal age (CPNA) 28 days (equivalent to early childhood in humans), glucose tolerance tests were performed, followed by euthanasia and collection of tissues. The prevalence of NALFD at CPNA 28 days was investigated using a NAFLD activity score based on histological methods and stains to visualise steatosis (oil red o), fibrosis (Masson’s trichome) and inflammation (H&E) in the liver. To observe hepatic alterations at a molecular level, amino acid profiles were assessed by liquid chromatography-tandem mass spectroscopy (LC-MS/MS).

**Results**: Lower BSL at birth was observed in preterms compared to terms (p=0.001), however, BSL was higher in preterms than term counterparts at TEA (p<0.001). Preterms had a significant increase in hepatic steatosis at CPNA 28 days (p=0.0428) Inflammation was not significantly increased in preterms (p=0.4823), and pathologic fibrosis was not observed in any individuals enrolled in the study. Decreased concentrations of essential amino acids associated with liver damage prevention were observed in preterms compared to terms, including tryptophan (p=0.0242) and leucine (p=0.0174). Preterm cohorts had increased concentrations of cysteine (p<0.001) an amino acid associated with hepatic steatosis.

**Conclusions**: Prematurity results in significant changes to hepatic metabolic function in the neonate. This deficit is reduced upon reaching term equivalent age, however molecular and physiological changes within the liver persisted into early childhood. In identifying metabolic, molecular and physiological alterations in the preterm guinea pig this study has begun to elucidate the pathways involved in preterm susceptibility to NAFLD and provided a solid platform for treatment.
### Abbreviations and Terms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>AUC</td>
<td>Area under curve</td>
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<tr>
<td>BMI</td>
<td>Body mass index</td>
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<tr>
<td>Boar</td>
<td>Adult male guinea pig</td>
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<tr>
<td>BRU</td>
<td>Biomedical research unit</td>
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<tr>
<td>BSL</td>
<td>Blood sugar level</td>
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<td>CPAP</td>
<td>Continuous positive airway pressure</td>
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<tr>
<td>CPNA</td>
<td>Corrected postnatal age</td>
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<tr>
<td>CRL</td>
<td>Crown - rump length</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
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<tr>
<td>Dam</td>
<td>Guinea pig mother</td>
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<tr>
<td>DIA</td>
<td>Digital image analysis</td>
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<tr>
<td>DOHaD</td>
<td>Developmental origins of health and disease</td>
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<td>FFA</td>
<td>Free fatty acids</td>
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<tr>
<td>FLI</td>
<td>Fatty liver index</td>
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<tr>
<td>FOV</td>
<td>Field of view</td>
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<tr>
<td>FWG</td>
<td>Fractional weight gain</td>
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<tr>
<td>GA</td>
<td>Gestation age</td>
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<tr>
<td>GTT</td>
<td>Glucose tolerance test</td>
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<tr>
<td>H&amp;E</td>
<td>Haematoxylin and eosin</td>
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<tr>
<td>HCC</td>
<td>Hepatocellular carcinoma</td>
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<tr>
<td>HL</td>
<td>Hind Limb</td>
</tr>
<tr>
<td>HT</td>
<td>Hock-Toe</td>
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<tr>
<td>IM</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IUUGR</td>
<td>Inter-uterine growth restricted</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun terminal kinas</td>
</tr>
<tr>
<td>LBW</td>
<td>Low birth weight</td>
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<tr>
<td>MetS</td>
<td>Metabolic syndrome</td>
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<tr>
<td>NAFL</td>
<td>Non alcoholic fatty liver</td>
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<tr>
<td>NAFLD</td>
<td>Non alcoholic fatty liver disease</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NAS</td>
<td>NALFD activity score</td>
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<tr>
<td>NASH</td>
<td>Non alcoholic steanohepatitis</td>
</tr>
<tr>
<td>ORO</td>
<td>Oil red o</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive end-expiratory pressure</td>
</tr>
<tr>
<td>PI</td>
<td>Ponderal Index</td>
</tr>
<tr>
<td>RIA</td>
<td>Radio immuno precipitation assay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SC</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Sire</td>
<td>Guinea pig father</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TEA</td>
<td>Term equivalent age</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral adipose tissue</td>
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1 Introduction

1.1 Premature Birth

1.1.1 Premature birth in New Zealand

Preterm births account for 7.4% of New Zealand’s birth rate, with those born to Maori and low socio-economic mothers being overrepresented. While the immediate effects detrimental to the premature neonate’s survival are widely recognized, there can be further complications that arise during the juvenile and adult stages of life that are less understood. Our understanding of the later-life effects, which stem from the developmental origin of preterm in utero growth have been shown to increase risk of cardiovascular disease, diabetes and components of metabolic syndrome in adults.

1.1.2 Later life complications of premature birth

Due to advances made in neonatal care 45 years ago we are now seeing the first wave of moderately premature children enter middle age and along with them the first signs of serious complications as a result of their altered developmental environment. In 1972 Liggins & Howie trialed the use of corticosteroids in human pregnancies where birth before 37 weeks was deemed likely. Antenatal betamethasone administered to accelerate lung maturation in the fetus was a significant improvement in neonatal care, significantly increasing the survival rate of premature babies below 32 weeks gestation. The trial would later become known as the ‘Auckland Steroid Study’. Thirty years later, Dalziel et al. investigated cardiovascular risk factors associated with preterm birth using the ‘Auckland Steroid Study’ cohort and demonstrated that gestational age was associated with increased systolic blood pressure and insulin resistance in adult life. This indicated that preterm birth has effects upon the individual that become apparent in later life. In Norway, Moster et al. used a similar cohort of premature babies to investigate the association of preterm birth with measures of wellbeing in adulthood. Medical and social criteria found to be associated with gestation age included mental retardation, education level, income, and receipt of disability or social security benefits. They concluded “medical and social disabilities in adulthood increase
with decreasing gestational age”. Studies such as these provide evidence of the long term challenge increased survival of premature babies confers; an expanding burden on national healthcare.

1.1.3 The effect of preterm later life complications on the health system
As the survival of premature babies increases thanks to advancements in antenatal treatments and neonatal care, ex-preterm infants constitute an increasing proportion of the population. This is especially true for the Maori and low socioeconomic population as they are overrepresented in the premature birth rate. An increased proportion of ex-preterm babies means an increased burden on the health system as they age and develop complications. It is therefore essential that studies into the long term effects of preterm birth continue, so treatments and preventative strategies can be devised to prevent the manifestation of such effects. One such long term complication is the increased risk of metabolic syndrome. Comprised of multiple dysfunctions, metabolic syndrome and its components have long been shown to be associated with premature birth.

1.2 Metabolic syndrome
Metabolic syndrome (MetS) is an aggregation of conditions including abdominal obesity, insulin resistance, hypertension, and atherogenic dyslipidemia, which greatly increase the risk of developing cardiovascular disease (CVD) and type 2 Diabetes Mellitus (T2DM). Furthermore, not all conditions have to be present to be diagnosed with MetS. Currently, a diagnosis of MetS occurs if an individual exhibits 3 of the major metabolic dysfunctions. An individual with MetS is 2 times more likely to develop CVD within 5-10 years than an individual without, 5 times more likely to develop T2DM, 3 times more likely to suffer a stroke or develop coronary heart disease. As such, risk of mortality as a result of experiencing a cardiovascular event is increased 1.8 times. Risk of developing CVD or T2DM is increased with the number of MetS conditions the patient suffers. Increases seen in patients with 2 or more conditions are beyond what would be expected if the conditions were presented independently, indicating interaction between the conditions which exacerbate their effects. Furthermore, additional risk factors for CVD and T2DM...
associated with MetS can include proinflammatory and prothrombotic states\textsuperscript{14}, hyperglycemia\textsuperscript{15}, Non-alcoholic Fatty Liver Disease (NAFLD)\textsuperscript{16}, endothelial dysfunction\textsuperscript{17}, and chronic stress\textsuperscript{18}. Development of MetS is largely attributed to obesity\textsuperscript{19}, with insulin resistance stemming from adipocyte dysfunction acting as a major etiological factor\textsuperscript{20,21}. Due to MetS’s association with obesity and insulin resistance, rates of MetS and associated dysfunctions are increasing worldwide\textsuperscript{22}. MetS is predicted to continue increasing in prevalence) and the hepatic manifestation of MetS, nonalcoholic fatty liver disease (NAFLD)\textsuperscript{23} is expected to become the leading etiology for liver transplantation in the 21st century\textsuperscript{24}. Liver dysfunction may play a key role in the development of MetS due to the liver’s role in maintaining metabolic homeostasis\textsuperscript{25}. It has been proposed that NAFLD and MetS share common pathophysiological mechanisms\textsuperscript{16}. Therefore the ability to determine a predisposition to NAFLD may provide an early indication of MetS susceptibility, opening the door for early intervention and treatment ahead of MetS manifestation.

1.3 Nonalcoholic Fatty Liver Disease

As the prevalence of MetS rapidly increases, so to has the occurrence of NAFLD. NAFLD’s close association with obesity results in rapidly increasing rate of the disease in developing and western countries where over nutrition and sedentary lifestyle\textsuperscript{26–28} are increasingly the norm. Between 1988 and 2008 rates of NAFLD in the United States doubled, while other causes of liver disease remained stable\textsuperscript{29}. NAFLD manifests analogously to Alcohol Fatty Liver Disease\textsuperscript{30}, in 1980 Ludwig \textit{et al}\textsuperscript{31} differentiated and entitled the disease through acknowledgement of its manifestation in the absence of excessive alcohol. As NAFLD can be asymptomatic\textsuperscript{32}, rates of diagnosis may be underestimated. When NAFLD is suspected, the gold standard for diagnosis requires liver biopsy, an invasive, risky and costly procedure\textsuperscript{32}; therefore biopsy is requested only when NAFLD is suspected and likely to result in complications. Non-invasive measures are limited in their sensitivity and staging abilities\textsuperscript{33}. A method of reliable non-invasive diagnosis is sought after as early detection prior to NAFLD development would be vastly beneficial in its allowance for intervention before disease development.
1.3.1 NAFL v NASH development

NAFLD is a spectrum disorder, comprised of two subtypes, nonalcoholic fatty liver (NAFL) - consisting of isolated steatosis, and nonalcoholic steatohepatitis (NASH) - characterised by hepatocellular injury and inflammation which may or may not be accompanied by fibrosis. NASH is considered to be the progressive form of NAFLD while NAFL until recently, was considered benign with little risk of progression to cirrhosis, hepatocellular carcinoma (HCC), hepatic failure, or mortality. A follow-up study of patients histologically diagnosed with NAFLD 18 years prior concluded progression rates were not uniform across the NAFLD spectrum. All NAFLD cases (including NASH) had a 22% risk of progressing to unfavourable outcomes, while NAFL cases had only 4% risk. In line with this, meta-analysis of paired biopsies taken 1 year apart revealed a subgroup within NAFL patients who had experienced rapid fibrosis progression. Further research is required to define the mechanisms surrounding this acceleration in fibrosis development, but Singh et al. postulate the difference in progression maybe due to inflammation in these NAFL cases which had not resulted in steatohepatitis cellular characteristics. While isolated liver steatosis does not in itself confer an increased rate of mortality, the associated conditions, CVD and T2DM do. Further, an individual with NAFL has a 4% risk of progression to NASH and with that the risk of progression to HCC and cirrhosis.

1.3.2 Manifestation of NAFLD in the liver

NAFLD incorporates 3 hepatic injuries, varying degrees of which confer NAFL or NASH (Figure 2, Figure 3, Figure 4).

**Steatosis** - Lipid accumulation within the liver has been credited to an imbalance in four distinct mechanisms: excess triglycerides are stored within the hepatocytes when the rate of free fatty acid (FFA) uptake and de novo lipogenesis, exceed the rate of lipid oxidation, and VLDL secretion. Steatosis may be a protective adaptation to limit the lipotoxicity of excessive FFA and lipid metabolites on the liver. Steatosis can manifests as micro- and macro-vesicular lipid deposits, or hepatocyte hypertrophy and ballooning when accompanied by inflammation (Figure 3).
**Inflammation** - Inflammation of the hepatocytes as a result of systemic (originating from adipose tissue dysfunction) and local inflammation of the liver $^{43}$. Inflammation manifests as hepatocellular ballooning and white blood cell infiltration $^{44}$ and can result in cellular degeneration and apoptosis $^{45}$ (Figure 4a).

**Fibrosis** - Fibrosis is a potential outcome of hepatocyte degeneration and apoptosis. Inflamed cells release cytokines which stimulate fibrogenic cells to produce collagen $^{46}$. Fibrosis manifests as an aggregation of extracellular collagen fibres occurring where damaged hepatocytes once existed. Fibrosis can impede liver function, with advanced hepatic fibrosis known as cirrhosis $^{47}$ (Figure 4b).
Figure 1: Multifactorial Pathogenesis of NAFLD resulting in NAFL or NASH

Predisposition through genetic, epigenetic and dietary factors may lead to obesity and insulin resistance which act upon the adipose tissue to encourage adipose dysfunction, pro-inflammatory cytokine release and lipolysis. Adipose tissue lipolysis and hepatic de novo lipogenesis create a flux of FFA which contributes to lipotoxicity of the liver triggering hepatocellular inflammation and oxidative stress (mitochondrial dysfunction, reactive oxygen species (ROS) production, and endoplasmic reticulum (ER) stress) 56. As a protective measure, excessive FFA in the liver is synthesised into triglycerides, and stored within the hepatocytes (i.e. steatosis) 39. Genetic and environmental factors further influence the hepatic response to increased steatosis and inflammation, determining the manifestation as NAFL or NASH 35.
Figure 2: H&E stained healthy liver tissue

Figure 2: H&E stained healthy liver tissue – Liver tissue free from steatosis and inflammation \(^{48}\).

Figure 3: H&E stained manifestations of hepatocellular steatosis at various magnifications

Figure 3: H&E stained manifestations of hepatocellular steatosis at various magnifications (A) Macrovesicular steatosis – singular large vacuoles displacing the hepatocyte nuclei. (B) Microvesicular steatosis – small vacuoles of lipid within the hepatocytes, arrow indicates a single hepatocyte with multiple lipid vacuoles. (C) Hepatocellular ballooning – enlargement of the hepatocyte (hypertrophy) with cytoplasmic degeneration indicated by arrow \(^{49}\).

Figure 4: Hepatic manifestation of fibrosis and inflammation

Figure 4: Hepatic manifestation of fibrosis and inflammation. (A) Inflammation foci within liver hepatic tissue, white blood cell infiltration into the liver visible in dark purple \(^{50}\). (B) Presinusoidal fibrosis of the liver, collagen fibres are visible in blue \(^{51}\).
1.3.3 The role of visceral adiposity and insulin resistance in NAFLD development

The impaired response to normal levels of insulin is key in the development of NAFLD: Insulin resistance associated with MetS and NAFLD is triggered by visceral obesity and the resulting dysfunction and inflammation of this adipose tissue. Oxygen supply to the increasing adipose tissue is limited due to hyperplasia and hypertrophy of the accumulating adipocytes, resulting in inflammation, apoptosis, and the release of FFA and adipokines (cytokines (cell signalling proteins) secreted by adipose tissue). These adipokines act locally through autocrine and paracrine signalling on the adipose tissue itself, and systemically, through endocrine signalling of the skeletal muscle, liver, and pancreas to alter insulin sensitivity. Adipokines identified to play a key role in the manifestation of NAFLD and insulin resistance include:

- **TNFα** - reduces insulin sensitivity by attenuation of ‘insulin receptor pathway 1’ in adipocytes.
- **IL-6** - chronically increased levels of IL-6 confer insulin resistance in liver tissue via activation of inhibitory SOCS-3 proteins.
- **PIA-1** - associated with obesity and predictive of T2DM development, however suspected indirect effects on insulin sensitivity have not been fully elucidated.

Lipolysis as a result of adipose dysfunction and insulin resistance increases circulation of FFA. Santomauro et al demonstrated a temporary lowering of insulin resistance in both nondiabetic obese and diabetic obese patients when Acipimox was administered to reduce FFA levels, thus implicating FFAs as a causal element in insulin resistance. This is achieved via the inhibition of insulin dependent glucose uptake of skeletal muscles, and increasing gluconeogenesis in the liver. Acute levels of FFA induce insulin secretion, while longer periods of FFA elevation impair insulin production. A positive feedback loop is created between insulin resistance and FFA release. Insulin’s suppression of adipose lipolysis is reduced with insulin resistance, increasing FFA circulation and amplifying insulin resistance and hyperglycemia. Cascading
insulin resistance is key in development of the oxidative stress, lipotoxicity and proinflammation required for NAFLD development 45.

**Proinflammation and Oxidative Stress** - FFA amplified through insulin resistance alters hepatocyte mitochondrial structure and function to increase production of toxic metabolites and ROS - worsening mitochondrial damage and promoting hepatocyte inflammation and apoptosis 66. The Unfolded Protein Response (UPR) is activated in the endoplasmic reticulum through mitochondrial stress and hyperglycemia 35. UPR triggers c-jun terminal kinase (JNK) mediated inflammation and apoptosis 67. Inflammation within the liver stimulates cytokine release which along with the adipocytes contribute to a proinflammatory state both locally in the liver, and systemically 43.

**Lipotoxicity** is the accumulation of lipid intermediates in non-adipose tissue, such as the liver or skeletal muscle, leading to cellular dysfunction or death. In the context of NAFLD, insulin resistance and hyperglycaemia encourage de novo lipogenesis of FFA from carbohydrates stored in the liver 68. Then, in what is thought to be a protective pathway, this excess FFA is esterified into triglycerides and stored within the hepatocytes 39.

1.4 Developmental origins of NAFLD and metabolic syndrome

The ‘Developmental Origins of Health and Disease’ paradigm (DOHaD) 69,70 describes the link between environmental conditions during development and long term health outcomes. Heerwagen et al 71 detail multiple studies in which DOHaD is supported: manifestation of obesity and metabolic diseases in offspring of obese, overweight or diabetic mothers implicates maternal lipidemia, glycemia and inflammation as potential developmental origins of epigenetic modifications leading to later life metabolic complications. As an adaptive response, epigenetic alterations may occur in response to in utero or early developmental conditions. Sub-optimal conditions can result in structural and functional changes in adipose and hepatic tissue, and may result in a predisposition to MetS and NAFLD 72,73.

Conversely, similar metabolic complications arise as the result of fetal undernutrition and Intrauterine growth restriction. By studying activation of hepatic nutrient sensor proteins in the offspring of nutrient
restricted rodents, Wolfe et al. concluded that fetal undernutrition can result in the reprogramming of sensor proteins. Normal nutritional load made available postnatally was then recognised as excess nutrition, priming pathways for hepatic lipid deposition. ‘The Thrifty Phenotype’ hypothesis describes such responses as an adaptive measure, preparing the fetus for an environment of low nutrition. However when the postnatal environment is one of stable or overnutrition, these responses become maladaptive, contributing to insulin resistance and create a predisposition to MetS.

Studies of low birth weight (LBW; birth weight below 2500g) cohorts appear to support this hypothesis. Human babies with LBW due to intrauterine growth restriction (IUGR) were shown to have a higher occurrence of pediatric NASH, while liver enzymes associated with hepatocellular injury were increased in women who were born LBW, when tested at 60-79 years of age. LBW and premature individuals undergo a compensatory rapid growth pattern after birth. Leunissen identified the first 3 months of infancy to be the crucial period in which weight gain increased visceral adiposity and insulin resistance in young adults. In 2014 Breij et al. used a Fatty Liver Index (FLI) to indicate risk of NAFLD development and determined rapid catch up growth in LBW babies was associated with NAFLD, while slow growth catch up and LBW alone were not. These studies demonstrate the influence of suboptimal intrauterine environment on the physiology and functionality of the offspring which can result in metabolic complications during adulthood. The thrifty phenotype may provide insight into the mechanisms which create a predisposition to MetS and NAFLD in ex-premature adults. Additionally, as premature babies typically experience a nutrient rich environment and increased weight gain as neonates, the ‘catch up’ early developmental environment may constitute a predisposition to NAFLD development.

1.5 Premature birth associations with NAFLD and metabolic syndrome

1.5.1 Human trials

Metabolic abnormalities have been found to be associated with preterm birth in human trials, including insulin resistance, T2DM, and hypertension. Meta-analysis of 27 studies into preterm adult
associations with metabolic dysfunctions indicated significant differences in term and preterm adult blood pressure and plasma low-density lipoprotein. A significant difference in insulin resistance between preterm and full term adults was not found due to conflicting results of the analysed studies. Studies on NAFLD association were not included for analysis. Investigations into adiposity of preterm babies at full term equivalent age using Magnetic Resonance Imaging, found altered adipose tissue distribution, with a significant increase in visceral adiposity. The same research group characterised an adult preterm phenotype in which adiposity was increased relative to full term counterparts, while BMI and other signs of overt obesity remained similar between the groups. Visceral abdominal adiposity was significantly increased in preterm-born men. As abdominal obesity is a key constituent, and possibly causal factor of MetS, this could mean preterm men are more vulnerable to its development. This study observed young adults (18-27 years old), with the authors postulating that the preterm phenotype would become more pronounced with age, as would metabolic dysfunction. Worryingly, these prematurely born adults may be missing out on required cardiovascular and metabolic interventions, as the lack of overt obesity while carrying excessive visceral fat at a young age is not the typical presentation of MetS.

Intrahepatocellular lipid deposition has been observed in studies of human preterm birth. Increases have been found in preterms at full term equivalent age and as young adults. Supporting the DOHaD and thrifty phenotype hypotheses, two studies undertaken in recent years observe the relationship between NAFLD and aspects of the postnatal environment of preterms. Vasu et al found that lipid intake in the first week after preterm birth is positively correlated with hepatocellular lipid deposition at term age. Breij et al determined accelerated weight catch-up in the first 3 months after term equivalent age was associated with increased FLI at 21 years of age. Both studies implicate early life nutrition as a factor of NAFLD predisposition.

NAFLD disease progression evolves throughout the life course of a human. While studies have observed differences in predisposition and lipid deposition in infancy, investigating NAFLD progression requires intervals of 20-60+ years in human trials. Animal trials allow for observation of NAFLD progression to
occur in an expedited timeframe due to swift disease progression within a shorter lifespan. Animal trials also afford the opportunity to maintain consistent environments across treatment groups, limiting variables inherent in human trials.

1.5.2 Animal trials

There are no published studies of NAFLD development in animal models after preterm birth, however animal trials in which *in utero* and early developmental conditions were manipulated may provide insight into potential pathways of NAFLD development in the preterm phenotype. High fat diets given to pregnant non-human primates, mice, and guinea pigs, have resulted in increased hepatic steatosis in offspring. Conversely, undernutrition of pregnant sheep increased steatosis in offspring, implicating a predisposition to NAFLD through “metabolic thriftiness”. Similarly, when testing lipogenic indices, Yamada *et al* suggested IUGR rat neonates and fetuses were likely to have developed distinct metabolic pathways resulting in additional lipid storage.

Parallels in the mechanisms of IUGR and undernutrition that lead to NAFLD may exist in the preterm phenotype, however specific investigation into the preterms association with NAFLD is required to understand the etiology and progression of the disease.

1.6 Identification and characterisation of NAFLD

1.6.1 Histology techniques

Currently, the gold standard for the diagnosis of NAFLD is histological analysis of liver biopsy. Specialty staining is used to visualise steatosis, inflammation and fibrosis characteristics of hepatic tissue. Previous NAFLD studies have used varying combinations of; hematoxylin and eosin (H&E), sirius red, oil red o (ORO) periodic acid schiff (PAS), Sweet’s reticulin and Masson’s trichrome to reveal metabolite deposition (H&E, ORO, PAS), fibrosis (Masson’s trichrome, sirius red, Sweet’s reticulin), and inflammatory cell infiltration (H&E) in the liver. The use of ORO is considered far superior to H&E in determining steatosis and triglyceride content, especially when used in conjunction with Direct Image Analysis (DIA). ORO adheres to lipid deposits to stain them unique to the background liver tissue, while H&E is only able to
show where lipid deposits previously resided as alcohol dissolves the lipid deposits during processing. Other spaces absent of stain (such as lumen and extracellular spaces) may be confused for lipid deposits especially when incorporating DIA. H&E was especially unsatisfactory in the visual determination of microvesicular steatosis, as droplets <15µm in size are near impossible to detect through visual determination alone.

1.6.2 Steatosis quantification
In comparing ORO and H&E staining, Levene et al. 42 found when determining the percentage of cells with microvesicular and macrovesicular lipid deposits visually, the sensitivity of ORO was so great that 90 - 100% of hepatocytes were classed as containing lipid deposits. This lead to an inability to differentiate the degree of steatosis when applying NAS, as all samples scored highly. However when analysing the % area of lipid deposits (as with DIA) and applying NAS, ORO was highly correlated with triglyceride levels. The study concluded with a strong recommendation that when possible ORO with DIA should be used to quantify steatosis.

1.6.3 Quantification of NAFLD using the NAFLD Activity Score
To quantify the degree of NAFLD within the liver samples, an activity score is applied to histology specimens. Brunt et al. 44 developed a NAFLD scoring system primarily to grade and stage NASH. This technique used the constellation of steatosis and fibrosis lesion features to determine a Necroinflammatory Grade and Fibrosis Score. When attempting to determine the probability of NAFLD progression to cirrhosis and CCH, Matteoni et al. 34 defined a spectrum of states incorporated within NAFLD in addition to NASH. Improved methods to stage the spectrum of NAFLD were designed and validated by Kleiner et al. 41. They proposed a NAFLD Activity Score (NAS) which used 3 criteria: Steatosis, Lobular inflammation and Ballooning to determine the severity of NAFLD. Fibrosis is scored separately as it is considered a result of NAFLD and by nature is less reversible.

1.7 Conclusion
Visceral obesity and insulin resistance leading to FFA flux and cytokine release result in lipotoxicity, oxidative stress and inflammation which manifest as steatosis, inflammation and fibrosis within hepatic
tissue. Predisposition to NAFLD or NASH development is multifactorial, including genetic, epigenetic and developmental factors.

Long term metabolic dysfunction as a result of premature birth is a growing area of concern as the increased survival rates of premature infants and their later life complications is predicted to inflate the burden on the national health system. The mechanisms and progression of NAFLD in the premature phenotype is not fully elucidated, further research is required in which the use of animal models will be a valuable tool. Determination of an association between prematurity and NAFLD predisposition in the guinea pig has not yet been investigated and is the first step in developing novel methods of recognition and intervention of NAFLD progression.
2 Methods

2.1 Animal care and husbandry

All animal work was performed in line with the ethical standards and guidelines laid out in Guide for the Care and Use of Laboratory Animals, 8th Edition, and the National Animal Ethics Advisory Commission, New Zealand. All work was performed with the prospective approval of the University of Otago Wellington Animal Ethics committee. All animals derive from the outbred colony of Dunkin Hartley guinea pigs maintained in the University of Otago – Wellingtons’ Biomedical Research Unit (BRU).

2.1.1 Housing

Guinea pigs were housed within open floor pens within a temperature (22°C) and humidity (50%) controlled facility. Sound insulated rooms in which the pens were contained were under a 12-hour day/night light cycle. Floor pens were covered in pussydoo® compressed wood cat litter (Azwood Ltd, New Zealand) and contained cardboard boxes and tubes to provide environmental enrichment. After weaning, and if not enrolled in the breeding program, all guinea pigs were housed in single sex rooms.

2.1.2 Feeding

Guinea pigs were feed a diet consistent with the nutritional needs of laboratory guinea pigs. Guinea pig pellets (Sharpes stock feeds, Carterton, New Zealand), hay (Sharpes Feed and Barn, Lower Hutt, New Zealand), fresh leafy vegetables and carrots (Jina’s Produce, New Zealand), were supplied daily. Millipore filtered water was supplied for drinking ad libitum. As guinea pigs are unable to synthesise vitamin C, dietary yield from fresh vegetables was supplemented by adding ascorbate acid (Sigma Aldrich, Sydney, Australia) to the water supply at a dose of 1.2g/L.

2.1.3 Breeding

Guinea pigs enrolled into the BRU’s breeding programme are housed in 6 separate harems consisting of a single boar and multiple (4 – 15) sows to ensure outbreeding in the colony. Female offspring enrolled as breeders are systematically assigned to a harem (lineage) to which they were not born. New stock sourced from external guinea pig facilities is brought in to the breeding programme every 3 years to limit inbreeding.

2.1.3.1 Post-partum mating

As breeding sows are housed with males throughout their oestrous cycle post-partum mating is integrated to give a reliable conception date of subsequent litters. As dams enter oestrous immediately after giving birth, allowing the dam to remain in the harem for 24 hours after delivery allows a short window for
conception for the dam’s next litter. Upon confirmation of pregnancy the gestational age and due date of the forthcoming litter is then known.

2.1.3.2 Confirmation of pregnancy through ultra-sound

All breeding sows receive an abdominal ultrasound weekly to check for pregnancy. To prepare for the ultrasound, hair is removed from the sow’s abdominal region with hair clippers (Heinger, Switzerland). The ultrasound is administered by a trained technician at the BRU using a SonoSite ultrasound (Fuji Film, Australia). An estimate of litter size can be eluded from ultrasound 4 weeks into the pregnancy.

2.1.3.3 Selection and randomisation of dams for study

Only dams who conceived during post-partum mating were enrolled into this study. Dams were randomly selected to be enrolled for term or preterm delivery upon confirmation of pregnancy.

2.1.4 Term delivery and postnatal care

2.1.4.1 Vaginal birth of term pups

Dams randomly selected to deliver full term (control) pups received no manipulation, allowing spontaneous delivery at 69 days gestation. From 55 days gestation until birth, pregnant dams were weighed and their pelvic symphysis measured daily, with observation twice daily. Within 12 hours of delivery, pups were weighed, sexed, and biometric measurement taken (See 2.2 Collection of Biometric data).

2.1.4.2 Postnatal care of term pups

Pups and dams were re-homed together into the ‘maternity ward’ (a smaller pen allowing the pups easier access to their mother) where they will receive postnatal care until weaning. At weaning (day 21), pups were housed within a wood shavings (Wholesale Landscapes, Nelson, New Zealand) lined cage, which they will share with no more than 5 weaned pups until 28 days old.

2.1.5 Preterm delivery and postnatal care

2.1.5.1 Inducing vaginal birth of preterm pups

Dams assigned to deliver preterm pups underwent pharmacological induction of labour at 62 days gestation. To stimulate lung maturation in the premature pup, dams received Betamethasone (1mg/kg; Merck, Sharp, & Dohme, New Zealand) injected subcutaneously (lml luer-lock™ syringe (Terumo, Philippines), 32g x 1” needle (Terumo) 48 hrs and 24 hrs prior to induction. Preterm labour was induced pharmaceutically with Aglepristone (10mg/kg, Provet, New Zealand) injected subcutaneously (10mg/kg;
1ml luer-lock™ syringe, 32g x 1” needle) 24hrs and 1hr prior to induction to terminate pregnancy and oxytocin (3IU/kg; Provet,) injected intramuscularly (1ml luer-lock™ syringe, 36g x ½” needle into alternating quadriceps of the dam to stimulate uterine contractions. Oxytocin injections were repeated every ½ hour for the first hour of the induction process, then every 15 minutes until delivery of the first pup. Oxytocin was then administered at 10 minute intervals following delivery of each pup or placenta. Oxytocin administration continued until all pups and placentas have been expelled. The dam is then placed in incubator with pups for observation.

2.1.5.2 Resuscitation of preterm pups

Upon birth the pup was dried and tactile stimulation applied with a hand towel, once breathing or gasping was observed, pups received continuous positive airway pressure (CPAP) respiratory support, which was administered at a 30:70 ratio of O₂ and medical air (BOC gasses, New Zealand) at a rate of 5L/min and titrated to 100% O₂ as necessary to achieve adequate oxygenation. This was delivered to pups through a small mask held tightly over the nose and mouth to create a seal (Neopuff Infant T-piece resuscitator, Fisher & Paykel Healthcare, New Zealand), with positive end-expiratory pressure (PEEP) of 5cmH₂O to establish and maintain functional residual capacity (Figure 5a). When required, intermittent positive pressure ventilation was provided at a rate of 60 breaths per minute with peak inspiratory pressures of 12cmH₂O for 90 seconds or until consistent independent breathing was established and maintained. Once adequate respiratory function was achieved pups were further dried and placed into a towel lined cage within a Dräger 8000 human incubator (Drägerwerk AG & Co., Lübeck, Germany) set to 33°C with 35% humidity. Supplemental oxygen was delivered to the incubator at 1L/min for the initial 12 hours.

2.1.5.3 Postnatal care of preterm pups

Approximately 1 hour after birth - once respiratory support was no longer required, pups were weighed, sexed, and their biometric measurements and blood sugar levels taken (See 2.3.4 Blood sugar level readings). To prevent dehydration, subcutaneous saline was administered (1ml luer-lock™ syringe, 32g x 1” needle) to pups 2 and 10 hours after birth or when signs of dehydration were observed (minimal urine output, reduced skin turgidity).

Hand feeding of the pups began approximately 2 hours after birth (Figure 5c). Milk supplement was warmed to approx. 37°C and administered orally with a 1ml syringe (Terumo). The amount of supplement given was dependent on the pup’s interest in the food, typically pups consumed <0.5ml at birth increasing to 2 – 3ml by term equivalent age. Details on the time, quantity and type of supplement given was recorded. As the pups were housed with their mother during this time, maternal milk gradually became the dominant source of nutrition as the pups became stronger and developed the ability to suckle.
Prior to each feeding (Table 1), pups were weighed and stimulated to evacuate their bowels and bladder. A cotton bud (Cotton tips, Protec) is used to apply lubricant (KY Jelly, Johnson and Johnson, USA) in a repetitive circular motion to the pup’s anogenital region (Figure 5d). Weights and evacuations are recorded.

Neonate pups remained in the incubator with their dams for 3-4 days post-delivery (Figure 5b). Temperature and humidity were titrated to achieve adequate body temperature and cages were changed daily to ensure a clean environment. During this time pups are closely monitored as the non-ambulatory pups are at risk of suffocation (through the dam pinning down her pups) and asphyxiation (from aspiration of food post-feeding).

Once pups are able to adequately maintain body temperature, pups and dam are moved from the incubator to an ambient environment (usually 3-4 days after delivery). The towel lining of the cage is replaced with wood shavings (Wholesale Landscapes, Nelson, New Zealand) once the pups are ambulatory. Upon reaching term equivalent age (TEA; 7 days after delivery; CPNA day 0) pups were rehomed to the ‘maternity ward’ alongside other dams and their litters, where they remained until weaning at CPNA Day 21. From TEA onwards, there is no difference in the treatment of term and preterm pups.
Table 1: Preterm pup feeding schedule

<table>
<thead>
<tr>
<th>Corrected postnatal age (Days)</th>
<th>Supplement</th>
<th>Feeding Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>-7 (birth)</td>
<td>Colostrum</td>
<td>2 hourly</td>
</tr>
<tr>
<td>-6</td>
<td>Guinea pig milk</td>
<td>3 hourly</td>
</tr>
<tr>
<td>-5</td>
<td>Guinea pig milk</td>
<td>3 hourly</td>
</tr>
<tr>
<td>-4</td>
<td>50:50 guinea pig milk and apple baby food</td>
<td>6 hourly*</td>
</tr>
<tr>
<td>-3</td>
<td>50:50 guinea pig milk and apple baby food</td>
<td>6 hourly*</td>
</tr>
<tr>
<td>-2</td>
<td>50:50 guinea pig milk and apple baby food</td>
<td>8 hourly*</td>
</tr>
<tr>
<td>-1</td>
<td>50:50 guinea pig milk and apple baby food</td>
<td>8 hourly *</td>
</tr>
<tr>
<td>0 (TEA)</td>
<td>No feeding unless weight is lost</td>
<td></td>
</tr>
</tbody>
</table>

Colostrum (Wombaroo Food Products, Adelaide, Australia), Guinea Pig milk (Wombaroo Food Products), Apple babyfood (Watties, New Zealand).

*extra feedings were given if pups were not consistently gaining weight.
Figure 5: Postnatal care of preterm pups

(A) A newborn preterm pup receiving resuscitation support. (B) Incubated dam with preterm pups, shortly after delivery. (C) A preterm pup, -4 days of age being feed with milk supplement. (D) Stimulation of anogenital region to promote bladder and bowel evacuations.
2.2 Collection of Biometric data

All pups were weighed daily on an electronic scale (Vibra SJ, Shinko Denshi, Japan) and measurements of Crown - Rump Length (CRL), Hock-toe (HT) and Hindlimb Length (HL) are taken twice weekly on days -7 & -4 (preterms only) and CPNA days 0, 4, 7, 10, 14, 17, 21 and 28 with a flexible tape measure (Liberty®, London, UK) (See Appendix 7.1, Page 97, Figure 31). Postnatal weight gain, adiposity, and growth rates were determined through transformation of the individual’s anthropometric measurements.

2.2.1 Fractional weight gain (FWG)

A measure of average daily weight within a given period of time. Calculated using the method outlined by Patel et al.\textsuperscript{97} FWG was calculated for the first 7 days, (Day -7 to Day 0 for preterms, Day 0 – Day 7 for terms) and for the postnatal period (CPNA day 0 – CPNA day 21 for both preterms and terms). To exclude initial postnatal weight loss from the preterm period, FWG was calculated only for the days within the 7 day period in which weight was not lost.

\textbf{Equation 1: Fractional Weight Gain}

\[ \text{FWG} \left( \frac{mg}{g \text{ per day}} \right) = \left[ 1,000 \times \ln\left( \frac{W_n}{W_1} \right) \right] / \left( D_n - D_1 \right) \]

Where:
- \( W_n \) = weight at end time point (g)
- \( W_1 \) = weight at start time point (g)
- \( D_n \) = last time point of period (days)
- \( D_1 \) = first time point of period (days)

2.2.2 Ponderal Index (PI)

An estimate of adiposity in relation to length. Calculated for time points at which measurements were taken (Days -7, -4, (prems only) 0, 4, 7, 10, 14, 17, 21 and 28.). The sum of a guinea pig’s Crown-Rump Length (CRL) and Hind Limb (HL) was used as an approximation of the animal’s height.
Equation 2: Ponderal Index

\[ PI \text{ (kg/m}^3\text{)} = \frac{W}{L^3} \]

Where:
\[ W = \text{weight (kg)} \]
\[ L = \text{total length (m)} = \text{CRL} + \text{HL} \]

2.3 Collection of metabolic data

The following techniques were employed as an indicator of the metabolic function of the treatment groups. Tracking alterations in fasted BSL throughout the guinea pigs life and in response to a glucose challenge can indicate disruptions in insulin sensitivity and glucose metabolism. Plasma samples were retained for paired insulin and amino acid assay at a later date.

2.3.1 Longitudinal fasted blood sugar levels

Blood sugar levels and blood samples were taken weekly on days -7 (preterms only at 1 and 10 hours post-birth, non-fasted, prior to feeding) and at CPNA day 0, 7, 14, 21 and 28 following a 4 hour fasting period.

2.3.2 Oral glucose tolerance test

Oral glucose tolerance tests (GTTs) were administered on CPNA day 28 after 4 hours of fasting. Animals were maintained under heat lamps and the temperature of the room was maintained at 30˚C to ensure circulation of blood in guinea pig extremities. Baseline blood samples and blood sugar levels were collected prior to an oral dose of glucose (1000mg/kg). Blood collection and blood sugar levels were then taken 30mins, 1hr, 2hrs and 3hrs post glucose dose.

2.3.3 Blood collection

Guinea pigs were swaddled within a towel to restrain. Aural veins were pricked with CareSens 28G lancets (Pharmaco Ltd, Auckland, New Zealand), blood was allowed to form a small bead upon the ear, before collecting with a microvette (Sarstedt, Germany). In some cases, gentle pressure was applied to the guinea pig’s ear to encourage blood flow. Full microvettes (0.3ml) were centrifuged at 4000rpm for 15 minutes. At least 100ml of plasma was extracted from each sample and stored at -80˚C.
2.3.4 Blood sugar level readings

Midway through blood collection (See, 2.3.3: Blood collection) blood was collected on a CareSens N Testing strip (Pharmaco) and assessment of blood sugar levels was carried out using CareSens N Glucometer (Pharmaco).

2.3.5 GTT analysis

For each animal, fasted blood sugar levels in response to a GTT administered at CPNA 28 days were graphed against time to create a dose response curve (Figure 6). Graphed baselines were adjusted to the animal’s baseline BSL. Area under the Curve (AUC), peak height and delta peak height (the difference between baseline BSL and peak height) were analysed for each individual.

At each time point of the GTT concurrent plasma samples were collected for paired insulin assay. Radio Immuno Precipitation Assay (RIA) is to be performed by Professor David Kennaway at the University of Adelaide. This protocol is currently being optimised.
Figure 6: Example of an individual’s plotted glucose tolerance test

- Baseline fasted BSL is the BSL measured prior to glucose dose – represented by dashed blue line. Absolute peak is the highest BSL reached – represented by the purple line. Δ peak is the highest BSL reached minus the baseline BSL – represented by the red line. Area under curve is the total area beneath the plotted curve above baseline - represented by the grey hashed area beneath the plotted line.
2.4 Post mortem collection of tissues

2.4.1 Anaesthesia

Guinea Pigs were euthanized on Day 28 prior to tissue collection. To anesthetise the animal ketamine (40mg/kg, PhoenixPharm, New Zealand) and medetomidine (Domitor®, 0.5mg/kg, Pfizer Animal Health, New Zealand) were injected into the intraperitoneal cavity (IP) via a single 1ml luer-loc™ syringe and G32 x 1" needle. To ensure appropriate level of anaesthesia toe pinch and eyelid reflexes were tested.

2.4.2 Measurements

Post-mortem measurements (Head length, nose-rump, crown-rump length, hind-limb, hock-toe, head circumference, neck circumference, chest circumference, abdominal circumference) were taken post anaesthesia using a flexible tape measure.

2.4.3 Terminal blood collection

Approximately 5ml of blood was collected prior to post mortem via cardiac punch. A 10ml luer-loc™ syringe was used with a 22g x ½" precisionglide™ needle (BD, Singapore) to draw blood from the heart. Blood was placed into 10ml EDTA vacutainers® (BD) and centrifuged (4°C, 4000rpm for 12 minutes). Plasma, buffy coat and red blood cells were separated and stored at –80 °C.

2.4.4 Euthanasia

1ml of Pentobarbital (Provet) was administered by cardiac injection using a 10ml luer-loc™ syringe with a 22g x ½" precisionglide™ needle (BD). Death was confirmed by lack of heartbeat ahead of post mortem collection. Secondary death measures (decapitation and perforation of the diaphragm) were also performed.

2.4.5 Tissue collection

All dissection was performed with surgical instruments including skin scissors, internal scissors, non-toothed tweezers, scalpel, and scalpel blade (ProSciTech, Australia). All tissues were split into fixed and frozen samples. Frozen tissues were snapped frozen in liquid nitrogen and stored at -80 °C. Tissues for paraffin sectioning were fixed in 10% Neutral buffered formalin (Labserv) overnight at 4 °C, before being rinsed and stored in phosphate buffer (7.4.1, Page 100) at 4°C until processed for histology.

2.4.5.1 Liver collection and processing

A midline incision was made through skin and tissue layers from the upper chest to lower abdomen. This incision was then extended laterally to allow access to the body cavity. The liver was dissected away from
the body and weighed. The lower right lobe of the liver was dissected, placed in a 50 x 60mm resealable bag (OfficeMax®, New Zealand) and snap frozen in liquid nitrogen. The left lower lobe was excised and formalin fixed within a plastic cassette and 80ml specimen container (Interlab, New Zealand) (See appendix 7.3: Liver dissection and sampling, Page 99).

2.4.5.2 Visceral adipose tissue collection and processing

Visceral adipose tissue (VAT) surrounding the kidneys was dissected and weighed. VAT from the right kidney was snap frozen and VAT from the left was formalin (10% buffered neutral formalin) fixed within a plastic cassette and 80 ml specimen container. Both samples were retained for potential future analysis.

2.4.5.3 Subcutaneous adipose tissue collection and processing

Subcutaneous adipose tissue was sampled from dorsocervical fat pad atop the shoulder blades of the guinea pig. The skin above the fat pad was removed to allow access, and the fat pad dissected out and weighed. The fat pad was then bisected through the midline, the right side snap frozen, the left formalin fixed within a plastic cassette and 80ml specimen container. Both samples were retained for potential future analysis.

2.5 Histology

2.5.1 Tissue for paraffin sectioning

2.5.1.1 Specimen preparation

To blind histological analysis of the tissue samples, each subject was assigned a random number (random.org – random sequence generator).

From the formalin fixed liver tissue, a 5mm thickness cross section through the liver lobe was sampled. Tissue from the widest part of the lobe was excised (See appendix 7.3, Page 99) using a scalpel, and placed into a small histosette (Interlab) labelled with the subjects assigned random number.

2.5.1.2 Tissue processing

To prepare the tissue for paraffin sectioning, the tissue was dehydrated using alcohol, cleared of the alcohol through replacement with xylene, and infiltrated with paraffin wax. This process was performed automatically using a Tissue-tek® VIP processor (Sakura® Finetek, USA) on a 13 hour cycle (Table 2).
Table 2: Tissue Processing Protocol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Duration</th>
<th>Temperature</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin</td>
<td>2 hours</td>
<td>37 °C</td>
<td>Fixation</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>90% ethanol*</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Dehydration</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Clearing of ethanol</td>
</tr>
<tr>
<td>Xylene</td>
<td>1 hour</td>
<td>37 °C</td>
<td>Clearing of ethanol</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>30 minutes</td>
<td>60 °C</td>
<td>Infiltration of wax</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>30 minutes</td>
<td>60 °C</td>
<td>Infiltration of wax</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>30 minutes</td>
<td>60 °C</td>
<td>Infiltration of wax</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>30 minutes</td>
<td>60 °C</td>
<td>Infiltration of wax</td>
</tr>
</tbody>
</table>

Ethanol (Labserv), xylene (Labserv), Paraplast Plus® paraffin wax (McCormick™ Scientific, USA)

*See solution appendix: 7.2.2, Page 86

2.5.1.3 Embedding

Using a Tissue-Tek® Embedding centre (Sakura™) each sample was embedded within a (30x20mm) mould with paraffin wax heated to 60 °C. The embedded sample was cooled on a Tissue-Tek® Cryoconsole (Sakura™), before removal from the mould.

2.5.1.4 Microtomy

4μm thick tissue sections were cut using a Leica RM2235 microtome (Leica Biosystems, New Zealand) with feather S35 blades (Feather Safety Razor co., Japan) and floated on a 48°C flotation waterbath (Thermo Scientific, New Zealand) for collection onto StarFrost® microscope slides (Knittel Glass, Germany).

For each sample, 6 serial sections were taken from 3 tissue depths (levels) approximately 800μm apart (Table 3).
Table 3: Order of sections cut and the stains they were used for.

<table>
<thead>
<tr>
<th>Level 1</th>
<th>Level 2</th>
<th>Level 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x H&amp;E</td>
<td>1x H&amp;E</td>
<td>1x H&amp;E</td>
</tr>
<tr>
<td>2x spares</td>
<td>2x spares</td>
<td>2x spares</td>
</tr>
<tr>
<td>1x trichrome</td>
<td>1x trichrome</td>
<td>1x trichrome</td>
</tr>
<tr>
<td>1x spare</td>
<td>1x spare</td>
<td>1x spare</td>
</tr>
<tr>
<td>1x sudan black (5μm)</td>
<td>1x sudan black (5μm)</td>
<td>1x sudan black (5μm)</td>
</tr>
<tr>
<td>1x spare (5μm)</td>
<td>1x spare (5μm)</td>
<td>1x spare (5μm)</td>
</tr>
</tbody>
</table>

2.5.1.5 Staining

2.5.1.5.1 Haematoxylin and Eosin (H&E)

Staining was batched according to level to ensure distribution of potential batch effects across all samples. All batches were stained using the same solutions following the Otago Wellington Pathology Departments protocol (Table 4). Stained sections were mounted in Tissue-Tek® Glas Mounting Media (Sakura® Finetek) and coverslipped (22 x 40, Menzel-Gläser, Thermo Scientific).

Table 4: Haematoxylin & Eosin Protocol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Duration</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Deparaffinization</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Deparaffinization</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Clearing of xylene</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Clearing of xylene</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>3 minutes</td>
<td>Rehydration</td>
</tr>
<tr>
<td>Running H₂O</td>
<td>3 minutes</td>
<td>Rehydration</td>
</tr>
<tr>
<td>Haematoxylin*</td>
<td>12 minutes</td>
<td>Staining of nuclear detail</td>
</tr>
<tr>
<td>H₂O</td>
<td>x1 dip</td>
<td>Remove excess haematoxylin</td>
</tr>
<tr>
<td>Acid Alcohol *</td>
<td>x2 dips</td>
<td>Differentiation of haematoxylin</td>
</tr>
<tr>
<td>Running H₂O</td>
<td>10 minutes</td>
<td>Blueing of haematoxylin</td>
</tr>
<tr>
<td>Eosin*</td>
<td>4 minutes</td>
<td>Staining of cytoplasm</td>
</tr>
<tr>
<td>Running H₂O</td>
<td>x5 dips</td>
<td>Remove excess Eosin</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Clearing of alcohol</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Clearing of alcohol</td>
</tr>
</tbody>
</table>

*See solution appendix: 7.4.2, Page 100
2.5.1.5.2 Masson’s Trichrome

Staining was batched according to level to ensure distribution of potential batch effects across all samples. Batches were stained following a protocol modified from IHCWorld\(^98\) (Table 5). Stained sections were mounted in Tissue-Tek® Glas Mounting Media and coverslipped.

**Table 5: Masson’s trichrome staining protocol**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Duration</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Deparaffinization</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Deparaffinization</td>
</tr>
<tr>
<td>100% ethanol *</td>
<td>3 minutes</td>
<td>Clearing of xylene</td>
</tr>
<tr>
<td>100% ethanol*</td>
<td>3 minutes</td>
<td>Clearing of xylene</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>3 minutes</td>
<td>Rehydration</td>
</tr>
<tr>
<td>Running H(_2)O</td>
<td>3 minutes</td>
<td>Rehydration</td>
</tr>
<tr>
<td>Bouin’s solution*</td>
<td>1 hour at 60 °C</td>
<td>Fixation</td>
</tr>
<tr>
<td>Running H(_2)O</td>
<td>10 minutes</td>
<td>Remove excess Bouin’s solution</td>
</tr>
<tr>
<td>Weigert’s Iron haematoxylin*</td>
<td>10 minutes</td>
<td>Nuclear staining</td>
</tr>
<tr>
<td>Running H(_2)O</td>
<td>10 minutes</td>
<td>Blueing of haematoxylin</td>
</tr>
<tr>
<td>Distilled H(_2)O</td>
<td>x5 dips</td>
<td>Wash</td>
</tr>
<tr>
<td>Biebrich scarlet-acid fuchsin*</td>
<td>12 minutes</td>
<td>Cytoplasmic staining</td>
</tr>
<tr>
<td>Distilled H(_2)O</td>
<td>x5 dips</td>
<td>Wash</td>
</tr>
<tr>
<td>Phosphomolybdic-phosphotungstic acid *</td>
<td>12 minutes</td>
<td>Differentiation</td>
</tr>
<tr>
<td>Light green in 1% acetic acid*</td>
<td>20 minutes</td>
<td>Staining of collagen</td>
</tr>
<tr>
<td>Distilled H(_2)O</td>
<td>x5 dips</td>
<td>Wash</td>
</tr>
<tr>
<td>70% ethanol*</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>100% ethanol</td>
<td>3 minutes</td>
<td>Dehydration</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Clearing of alcohol</td>
</tr>
<tr>
<td>Xylene</td>
<td>5 minutes</td>
<td>Clearing of alcohol</td>
</tr>
</tbody>
</table>

*See solutions appendix: 7.4.3, Page 100
2.5.2 Tissue for frozen section

2.5.2.1 Specimen preparation and cryosectioning

From frozen liver tissue, a 5mm thickness cross section through the liver lobe was sampled. Tissue from the widest part of the lobe was excised (See appendix 7.3, Page 99) using a coping saw (Fuller, Canada) and placed into small plastic bag labelled with the subjects assigned random number.

Tissue was embedded within Tissue-Tek® O.C.T. Compound (Sakura®) within a Tissue-Tek® cryostat (Sakura®) set at -20°C. 12μm sections were cut using feather s35 blades and adhered to polycine® coated slides (Labserv).

2.5.2.2 Oil red O Staining

All samples were stained within the same batch following an oil red o protocol adapted from Melehem et al99 and Bancroft et al100 (Table 6). Stained sections were mounted in Shandon Immuno-Mount™ (Thermo Scientific) and coverslipped.

Table 6: Oil red O staining protocol

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Duration</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formalin</td>
<td>20 seconds</td>
<td>Fixation</td>
</tr>
<tr>
<td>Oil Red O *</td>
<td>5 minutes</td>
<td>Lipid staining</td>
</tr>
<tr>
<td>Harris’s Haematoxylin *</td>
<td>15 seconds</td>
<td>Nuclear staining</td>
</tr>
<tr>
<td>Running H₂O</td>
<td>30 minutes</td>
<td>Blueing of haematoxylin &amp; washing away excess ORO</td>
</tr>
</tbody>
</table>

*see solutions appendix: 7.4.4, Page 101

2.5.3 Histological analysis

2.5.3.1 Microscopy and photography

Microscopy was performed on an Olympus BX51 microscope with images captured using a Olympus DP20 mounted camera (Olympus, New Zealand).

Five fields of view at 100x or 200x magnification were photographed from each section (3 sections for each sample). These 5 fields of view were taken in the same general areas of each section (See appendix 7.3, Page 99) Randomization within these areas was ensured by positioning the slide upon the stage macroscopically - therefore ensuring microscopic details of the slide would not influence the area selected for sampling.
2.5.3.2 Quantification of hepatic physiology using NAFLD Activity Score (NAS)

To quantify the presence of NAFLD features (steatosis, inflammation, fibrosis) a NAFLD Activity Score (NAS) was applied to each sample. The scoring applied in this study was adapted from Kleiner et al’s system devised for quantifying the severity of NAFLD presentation in humans. Adoptions were based upon Liang et al’s murine and rodent NAS validation, and incorporated to increase sensitivity of detection as the NAFLD presentation was expected to be minor in our relatively young subjects.

2.5.3.2.1 Steatosis

Hepatocyte steatosis was assessed on H&E stained sections photographed at 100x magnification. Lipid droplet % coverage of each field of view was assessed using ImageJ version 1.51n (National Institutes of Health, USA) by measuring area fraction with colour threshold (Hue: 227 – 71, Saturation: 81 – 255, Brightness: 0 – 255). The average % coverage of the 5 fields of view of each sample was scored based on Kleiner et al’s criteria (Table 7).

Table 7: Kleiner et al’s NAS steatosis score criteria

<table>
<thead>
<tr>
<th>Score</th>
<th>Lipid droplet % area coverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt; 5%</td>
</tr>
<tr>
<td>1</td>
<td>5 – 33%</td>
</tr>
<tr>
<td>2</td>
<td>33 – 66%</td>
</tr>
<tr>
<td>3</td>
<td>&gt;66%</td>
</tr>
</tbody>
</table>

2.5.3.2.2 Inflammation

Lobular inflammation was assessed on H&E stained sections photographed at 100x magnification. Inflammatory foci in each field of view were counted to quantify inflammation. Inflammatory foci were considered to be a cluster (not a row) of 5 or more inflammatory cells within a 100 μm² area. Foci larger than 100 μm² were counted as a single foci. Inflammatory cells surrounding portal triads were excluded, as portal inflammation was not assessed. The average of the 5 fields of view of each level was scored based on Liangs et al’s rodent NAS criteria to give a score for the sample level and two inflammation scores were calculated for each subject:

1. The scores of the 3 levels of a given sample were then averaged to give an inflammation score for the subject.
2. The number of foci per field of view, averaged over the 15 fields of view from each subject.
Table 8: Liang et al’s’ Inflammation score system

<table>
<thead>
<tr>
<th>Score</th>
<th>Number of foci (at 100x mag)</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.5</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>0.5 – 1</td>
<td>Slight</td>
</tr>
<tr>
<td>2</td>
<td>1 – 2</td>
<td>Moderate</td>
</tr>
<tr>
<td>3</td>
<td>&gt; 2</td>
<td>Severe</td>
</tr>
</tbody>
</table>

Hepatocyte ballooning was assessed on H&E stained sections photographed at 100x magnification. Hepatocytes were described as ‘ballooned’ when enlarged beyond twice their original size, with flocculent cytoplasm. Ballooned hepatocytes in each field of view were counted to quantify inflammation. ‘Few’ was considered to be 10 or less ballooned cells within the field of view.

Table 9: Kleiner et al’s’ Hepatocyte ballooning score system

<table>
<thead>
<tr>
<th>Score</th>
<th>Extent</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Few ballooned cells</td>
<td>Rare but definite ballooned hepatocytes</td>
</tr>
<tr>
<td>2</td>
<td>Many balloon cells / prominent ballooning</td>
<td></td>
</tr>
</tbody>
</table>

2.5.3.2.3 Fibrosis

Hepatic fibrosis was assessed on Masson’s trichrome stained sections photographed at 100x magnification. The presence and location of collagen staining was used to determine the presence and severity of pathologic fibrosis using the fibrosis scoring system described by Kleiner et al (Table 10).
Table 10: Kleiner et al’s Fibrosis score system

<table>
<thead>
<tr>
<th>Score</th>
<th>Fibrosis location and extent</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>1</td>
<td>Perisinusoidal or periportal</td>
</tr>
<tr>
<td>1A</td>
<td>Mild (‘delicate’), Zone 3, Perisinusodial</td>
</tr>
<tr>
<td>1B</td>
<td>Moderate (‘dense’), Zone 3, Perisinusodial</td>
</tr>
<tr>
<td>1C</td>
<td>Portal / Periportal</td>
</tr>
<tr>
<td>2</td>
<td>Perisinusoidal and Portal / Periportal</td>
</tr>
<tr>
<td>3</td>
<td>Bridging fibrosis</td>
</tr>
<tr>
<td>4</td>
<td>Cirrhosis</td>
</tr>
</tbody>
</table>

2.6 Hepatic metabolomics analysis

2.6.1 Extraction

Amino acid analysis was performed by the Analytical Facility for Bioactive Molecules, The Hospital for Sick Children, Toronto Canada. Ground tissue was weighed out and a solution of 1:1 water : methanol was added to yield 25mg/mL. Samples were then homogenized and kept on ice. An equivalent of 5mg of homogenized tissue was added to an Eppendorf tube with an acylcarnitine internal standard (IS) mixture (NSK-B, Cambridge Isotopes, USA) and an amino acid IS mixture (Arginine-13C6, ADMA-d7, citrilline-d7, glutamic acid-d5, ornithine-d7, and leucine-d10, Cambridge Isotopes and CDN Isotopes, USA). Samples and standard mixtures were then acidified with 60µL of 0.1% formic acid and then protein was precipitated using 1mL of formic acid in acetonitrile. Tubes were then vortexed and centrifuged at 10,000g for 10 minutes at 4°C. Supernatants were transferred to conical glass tubes and the remaining pellet in the Eppendorf was re-suspended in an additional 1mL 0.3% formic acid in acetonitrile and vortexed and centrifuged again. The combined supernatants were dried under gentle flow nitrogen gas. Samples and standards were derivatized with 100µL butanolic-HCL3N for 30 minutes at 65°C. Solvent was then removed under gentle flow nitrogen gas and samples were reconstituted in 200µL MPS and transferred to autosampler vials. 10µL of the reconstituted sample was diluted 50x in the same solution for amino acid analysis.

2.6.2 Liquid chromatography-tandem mass spectroscopy (LC-MS/MS)

Extracted samples were injected onto Kinetex HILIC 50 x 4.6mm 2.6µ, column (Phenomenex, USA) connected to an Agilent 1290 HPLC system attached to a Q-Tarp 5500 mass spectrometer (AB Sciex, USA). Injected samples were eluted with a gradient of mobile phases MPA: 90/10 f mM ammonium formate pH
3.2/acetonitrile and MPB: 10/90 5mM ammonium formate pH 3.2/acetonitrile. The diluted samples were eluted with a gradient of MPA starting at 4% for 2.5 minutes and increasing to 75% MPA until 6 minutes, holding at 75% MPA for 1 minute and then returning to 4% over the final 2.5 minutes. Mass transitions were monitored for each sample and compared against known standard mass transitions for species analysed. Data was collected and analysed using Analyst v1.6 (AB Sciex).

2.7 Statistical Analysis

Statistical analysis of all data was performed using STATA version 15 (StataCorp, USA). The subjects received two treatments, full term birth and premature birth. Within these treatments the subjects were further divided into male and female groups to account for potential variation due to sexual dimorphism.

Data distribution within the 4 groups (term males, preterm males, term females, preterm females) was tested with Shapiro-Wilk test for normality and Levene’s robust test for equality of variances.

Comparisons between the four groups were made using a 2x2 factorial analysis of variance (2-way ANOVA) with the inclusion of interaction effects between prematurity and sex. A multi factorial design was chosen as it can account for a larger proportion of variability using smaller sample sizes (an ethical priority when using animal models) than single factor analysis allows. Longitudinal data was compared between cohorts of the same sex and assessed by random-effects GLS regression. Pearson correlations were used to determine strength and significance of associations between data sets. Significance was set at p<0.05.

Prism version 7 (Graphpad Software, USA) was used to generate figures. All data is presented as mean ± standard deviation to explain the variation between individuals that have contributed to the given mean.
3 Results

3.1 Cohort mortality and assignment

The rates of spontaneous stillbirth in the BRU’s guinea pig colony were at ~ 10% during the study period. Of live born term guinea pigs at the BRU there was a 3.9% mortality rate during the birth – weaning period. Within the 13 term litters produced for this study there were no stillborn pups, or pup deaths (Figure 7). Within the 15 preterm litters produced for this study, 18.03% of the 61 preterm pups produced were stillborn (n=11). 8% of live born preterm pups died during the preterm period (birth – TEA) (n=4). Of the 4 dead pups, 1 was euthanized due to significant respiratory distress, 3 were found dead - suspected to have been suffocated or crushed by their mother. All pups surviving the preterm period remained healthy until the studies end point at 28 days.

Male and female live born pups were randomly assigned to this (NAFLD) study and other concurrent studies. Siblings of the same sex were not enrolled within the same study group to prevent litter effect bias.

Figure 7: Allocation and mortality of term and preterm pups

Figure 7 – Allocation of live born term and preterm pups.
3.2 Biometrics

3.2.1 Weight

As expected, preterm birth had a significant effect on birth weight, with both male and female term pups weighing more than their preterm counterparts (p<0.001; term females, 91.7 ± 9.7g vs. preterm females 72.41 ± 4.7g, and term males, 95.3 ± 14.4g, vs. preterm males, 77.7 ± 6.5g; Figure 8a).

Weight loss immediately following birth is well described in both term and preterm infants. In the current study, preterm pups lost a significantly higher proportion of their body weight than term pups (p<0.001; term males: 0.4 ± 0.7% lost over 0.6 ± 0.9 days vs. preterm males: 9.5 ± 3.4% over 2.9 ± 0.4 days, term females: 0.5 ± 1.2% over 0.4 ± 0.9 days vs. preterm females: 8.6 ± 3.5% over 2.6 ± 0.5 days; Table 11).

Weight loss stabilised at approximately 3 days postnatal age (CPNA -5 days) in preterm animals (Table 11), following which both male and female preterm pups gained weight at rates comparable to that seen in term newborns over the first week of life (p=0.3427; term females: 59.3 ± 11.5mg/g/day vs. preterm females 68.2 ± 17mg/g/day; term males: 56.7 ± 14.4mg/g/day vs. preterm males 56.7 ± 9.3mg/g/day; Figure 10a). Over this period, preterm pups gained enough weight that there was no difference in weight between preterm animals at TEA and term birth weight (p=0.8204; Figure 8b).

Weight gain from CPNA day 0 (TEA in preterm pups) to weaning was not different between preterm and term animals (p=0.3885;Figure 10b). Preterm birth and sex therefore had no significant effect on weight at CPNA day 28. On average term males and females weighed heavier than their preterm counterparts (p=0.0715; term females, 263.9 ± 20.9g vs. preterm females 242.9 ± 33.8g; and term males, 281.9 ± 34g, vs. preterm males, 264.9 ± 27.4g Figure 8C). Both term and preterm males were heavier than term and preterm females at weaning (p=0.0587).

Growth trajectory was similar between term and preterm males until CPNA day 21 (weaning) (Figure 9). However, by CPNA day 28 significant divergence was observed, with a greater growth rate in term males than in preterms (p=0.003). In females, terms and preterms had similar growth rates between TEA and CPNA day 11. From CPNA day 12, terms and preterms began to diverge, with term females showing greater growth rate compared to preterms (CPNA day 12, p=0.05; CPNA day 13, p=0.04; CPNA day 14, p=0.05; CPNA day 15, p=0.03; CPNA day 16, p=0.01; CPNA day 17, p=0.08; CPNA day 18, p=0.03; CPNA day 19, p=0.003; CPNA day 20, p=0.003; CPNA day 21, p=0.07; CPNA day 28, p=0.003; Figure 9).
Figure 8: Weight of term and preterm pups

(A) Weight - Birth

(B) Weight - TEA

(C) Weight - CPNA 28 days

* denotes significance of p < 0.01.

Figure 8: Weight of term and preterm pups — Solid pink bars represent term females (n=9), hashed pink bars represent preterm females (n=7), solid blue bars represent term males (n=10), and blue hashed bars represent preterm males (n=8). (A) Birthweight (B) Weight at term equivalent age (TEA) (C) Weight at CPNA day 28. All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of p < 0.01.
Figure 9: Weight growth rate over time of term and preterm pups

Weight growth rate over time of term and preterm pups. Day -7 to CPNA day 28. Solid pink lines represent term females (n=9), dashed pink lines represent preterm females (n=7), solid blue lines represent term males (n=10), and blue dashed lines represent preterm males. All data analysed with random-effects GLS regression and presented as group means ± SD. * denotes significance of p < 0.05.
Table 11: Postnatal weight loss of term and preterm pups

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term (n=10)</td>
<td>Preterm (n=9)</td>
</tr>
<tr>
<td>Initial period of weight loss (days)</td>
<td>0.6 ± 0.9</td>
<td>2.9 ± 0.4*</td>
</tr>
<tr>
<td>Total weight lost</td>
<td>0.37g ± 0.6g</td>
<td>7.3g ± 2.9g*</td>
</tr>
<tr>
<td>Weight loss (% birth bodyweight)</td>
<td>0.4% ± 0.7%</td>
<td>9.5% ± 3.4%*</td>
</tr>
<tr>
<td>Daily weight lost</td>
<td>0.2g ± 0.3g</td>
<td>2.5g ± 0.9g*</td>
</tr>
</tbody>
</table>

Table 11: Postnatal weight loss of term and preterm pups—All data analysed using 2-way ANOVA and presented as mean ± SD. * denotes significance of p < 0.01.

Figure 10: Fractional weight gain of preterm and term pups

(A) Fractional weight gain - 1st 7 days ex weight loss

(B) Fractional weight gain - CPNA day 0 - 21

Figure 10: Fractional weight gain of preterm and term pups—solid pink bars represent term females (n=9), hashed pink bars represent preterm females (n=7), solid blue bars represent term males (n=10), and blue hashed bars represent preterm males (n=8). (A) FWG during first 7 days of life when initial period of weight loss is excluded (terms day 0-7, preterms day-7 = 0). (B) FWG from CPNA day 0 - day 28. All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of prematurity p < 0.01.
3.2.2 Length

Birth length (CRL) was significantly affected by prematurity, male and female term pups were both longer than their preterm counterparts (p<0.001; term females, 122 ± 10mm vs. preterm females 111 ± 4mm, and term males, 128 ± 8mm, vs. preterm males, 155 ± 6mm; Figure 11a).

By TEA, preterm and term males had grown to be significantly longer than their female counterparts (p=0.0089; term males, 128 ± 8mm, vs. term females, 122 ± 10mm, preterm males, 134 ± 4mm vs. preterm females 125 ± 7mm, Figure 11b). Preterm male and females had both grown in length to surpass term born animals, although not significantly. (p=0.0724).

Sex and prematurity had no effect on length at CPNA 28 days. Length was relatively consistent across all groups (prematurity p=0.4144, sex p=0.2330; term females, 192 ± 7.1mm, vs. preterm females, 190 ± 6.9mm vs. term males, 189 ± 8.3mm vs. preterm males 186±11mm; Figure 11c).

Linear growth trajectory was not affected by prematurity overall in male (p=0.100) or female (p=0.378) cohorts. However, within the males the preterm growth curve deviated from the terms’ from CPNA day 14 until the end of the study period (CPNA day 14, p=0.002; CPNA day 17, p=0.037; CPNA day 21, p=0.007; CPNA day 28; p=0.003; Figure 12). It is not known if this deviation would continue after the juvenile period/the studies endpoint. Female preterms deviated from their term counterparts even earlier than the males, displaying less CRL growth velocity than the terms from CPNA day 7 and convergence by the end of the study period (CPNA day 7, p=0.043; CPNA day 10, p=0.015; CPNA day 14, p=0.027; CPNA day 17, p=0.004; CPNA day 21, p<0.001; CPNA day 28, p=0.173; Figure 12).
Figure 11: Crown – Rump length of term and preterm pups

Solid pink bars represent term females (n=9), hashed pink bars represent preterm females (n=7), solid blue bars represent term males (n=10), and blue hashed bars represent preterm males (n=8). (A) Birth Crown-Rump length (CRL) (B) CRL at term equivalent age (TEA) (C) CRL at CPNA day 28 (D) Growth Rate (CRL) - day -7 to CPNA day 28. All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of prematurity p < 0.01.
Figure 12: Linear growth over time of term and preterm pups

Crown-Rump Length - Growth rate

-7  0  7  14  21  28
Age (days)

P overall male = NS
P overall female = NS

Term Male
Term Female
Preterm Male
Preterm Female

Solid pink lines represent term females (n=9), dashed pink lines represent preterm females (n=7), solid blue lines represent term males (n=10), and blue dashed lines represent preterm males. All data was analysed with random-effects GLS regression and presented as group means ± SD. * denotes significance of prematurity p < 0.05.
3.2.3 Adiposity as assessed by ponderal index

Surprisingly average adiposity at birth was slightly greater in preterm pups, although this was not significant (p=0.1178, term females, 22.8 ± 3.6kg/L³ vs. preterm females 23.5 ± 1.1kg/L³, and term males, 20.7± 2kg/L³, vs. preterm males, 22.8 ± 2.7kg/L³; Figure 13a).

There was a significant effect of prematurity at TEA, with adiposity of preterms reducing to below that of the terms (p=0.0289; term females, 22.8 ± 3.6kg/L³ vs. preterm females 20.4 ± 0.9kg/L³, and term males, 20.7± 2kg/L³, vs. preterm males, 19.4 ± 1.3kg/L³; Figure 13b). On average male adiposity remained lower than that of females pups (p=0.0697).

By the studies endpoint at CPNA day 28, adiposity was comparable between term females, term males, preterm males and preterm female (prematurity p=0.1012, sex p=0.6474; term males, 21.9 ± 1.4kg/L³, preterm males 21.7 ± 2.1kg/L³ and term females, 20.9 ± 2.1kg/L³, vs. preterm females, 23 ± 1.3kg/L³; Figure 13c).

Preterm females differed in their overall velocity of adipose development compared to term females (p=0.012) with an altered adipose development curve at all time points (CPNA day 3, p=0.044; CPNA day 7, p=0.007; CPNA day 10, p=0.012; CPNA day 14, p=0.028; CPNA day 21, p<0.001; CPNA day 28, p<0.001; Figure 14). On the other hand, prematurity in the males did not result in an overall effect on adipose development velocity (p=0.81), However divergence from term males did occur between their trajectories, with convergence by CPNA day 28 (CPNA day 3, p=0.001; CPNA day 10, p=0.034; CPNA day 14, p=0.001; CPNA day 21, p<0.004; CPNA day 28, p=0.111; Figure 14).
Figure 13: Adiposity (ponderal index) of term and preterm pups

(A) Ponderal Index (PI) at birth
(B) PI at TEA
(C) PI at CPNA day 28

All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of prematurity p < 0.01.
Figure 14: Adiposity development over time of term and preterm pups

Adiposity- Growth rate

Ponderal Index (g/mm³)

Term Male
Term Female
Preterm Male
Preterm Female

P overall male = NS
P overall female = 0.012

Age (days)

-7 0 7 14 21 28

Solid pink lines represent term females (n=9), dashed pink lines represent preterm females (n=7), solid blue lines represent term males (n=10), and blue dashed lines represent preterm males. All data analysed with random-effects GLS regression and presented as group means ± SD. * denotes significance of prematurity p < 0.05.
3.3 Metabolism

3.3.1 Weekly fasted blood sugar Levels

As described in humans, birth fasted BSL was significantly affected by prematurity, with lower BSLs recorded for male and female preterm pups than terms. (p<0.001; term females, 6.2 ± 1.1mmol/L vs. preterm females 4.4 ± 1.6mmol/L and term males, 6.4 ± 0.9mmol/L, vs. preterm males, 4.5 ± 1.8mmol/L; Figure 15a).

Upon reaching TEA, preterm metabolism had over-compensated by increasing fasted BSL to be significantly greater than the fasted BSL of the term cohorts. (p<0.001; term females, 6.2 ± 1.1mmol/L vs. preterm females 7 ± 1.6mmol/L and term males, 6.4 ± 0.9mmol/L, vs. preterm males, 8.5 ± 0.4mmol/L; Figure 15b). BSL of term pups also increased in during their first 7 days of life, however this was not to the same extent seen in the preterms (p<0.001; term females increased 0.8 ± 1.7mmol/L vs. preterm females 3.7 ± 2.5mmol/L, term males increased 1.2 ± 1.6mmol/L, vs. preterm males, 4.0 ± 1.8mmol/L; Table 12).

Neither prematurity nor sex had a significant effect on fasting BSL at CPNA day 7, as metabolism of the preterm animals had stabilised. On average term females had lower BSL than term males (term females, 6.9 ± 1.6mmol/L vs. term males, 7.5 ± 1.8mmol/L,) while BSL of the preterm cohorts were similar (preterm females, 6.6 ± 1.1mmol/L vs. preterm males, 6.4 ± 1.1mmol/L) (p=0.7087; Figure 15c).

Final fasted BSLs were obtained at CPNA day 28 where no significant differences were found between any of the cohorts. (p=0.0565; term females, 7.6 ± 0.5mmol/L, vs. preterm females, 7.3 ± 0.6mmol/L and term males, 7.9 ± 1.1mmol/L, vs. preterm males, 7.1 ± 0.5mmol/L; Figure 15d).

Both male and female preterms displayed deviations in overall BSL trajectory when compared to their term counterparts (male p<0.001, female p<0.001). BSL movement was altered in the preterms at all time points (males; CPNA day 7, p<0.001; CPNA day 14, p<0.001; CPNA day 21, p=0.001; CPNA day 28, p<0.001, females; CPNA day 7, p=0.002; CPNA day 14, p=0.004; CPNA day 21, p<0.001; CPNA day 28, p p=0.004; Figure 16). It is not known if convergence would have occurred beyond the study period/ the juvenile stage.
Figure 15: Weekly fasted blood sugar levels of term and preterm pups

(A) Birth blood sugar level (BSL)  (B) BSL at TEA (C) BSL at CPNA day 7  (D) BSL at CPNA day 28  (E) Weekly BSL - day -7 to CPNA day 28. All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of p < 0.01.
Figure 16: Blood sugar levels over time of term and preterm pups. Day -7 to CPNA day 28. Solid pink lines represent term females (n=9), dashed pink lines represent preterm females (n=7), solid blue lines represent term males (n =10), and blue dashed lines represent preterm males. All data analysed with random-effects GLS regression and presented as group means ± SD. * denotes significance of p < 0.01.
Table 12: Change in fasted blood sugar level during the 1st 7 days of life in term and preterm pups.

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term (n=10)</td>
<td>Preterm (n=8)</td>
</tr>
<tr>
<td>Change in BSL over 1st 7 days of life (mmol/L)</td>
<td>1.2 ± 1.6</td>
<td>4.0 ± 1.8*</td>
</tr>
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Table 12: Change in fasted blood sugar level during the 1st 7 days of life in term and preterm pups. - First 7 days of life occurs from CPNA day -7 to day 0 in preterms, day 0 – day 7 in term pups. All data analysed using 2-way ANOVA and presented as mean ± SD. * denotes significance of p < 0.01.
3.3.2 Glucose tolerance test

In addition to the fasted BSL taken at CPNA day 28, BSLs in response to a glucose tolerance test (GTT) were recorded and plotted (Figure 6, Page 24). There was no significant effect of sex or preterm birth on the pups’ baseline, absolute peak, or $\Delta$-peak BSLs during the GTT, neither was there an effect on AUCs generated from the GTT (Figure 17). Preterm birth and sex had no significant effect on peak BSL ($p=0.1802$; term males, $19 \pm 2.5$mmol/L, vs. preterm males, $18.3 \pm 3.6$mmol/L and term females, $17.6 \pm 1.5$mmol/L vs. preterm females, $15.7 \pm 1.4$mmol/L; Table 13). The same pattern was exhibited in $\Delta$-peak BSL ($p=0.6013$; term males, $11 \pm 1.8$mmol/L, vs. preterm males, $11 \pm 3.4$mmol/L and term females, $9.9 \pm 2$mmol/L vs. preterm females, $8.3 \pm 0.9$mmol/L; Table 13).

One male preterm was excluded from GTT analysis as the final BSL reading at 180 minutes was not collected. Of the 33 individuals with a complete GTT, 6 did not return to their baseline level within the time frame of the GTT. These individuals were term females ($x3$), preterm females ($x2$) and a term male ($x1$). These individuals were included in all GTT analyses as their exclusion did not affect statistical analyses.
Figure 17: Blood Sugar levels in response to glucose tolerance test at CPNA 28 days.

Blood sugar levels in response to GTT
CPNA 28 days

Time after glucose dose (mins)

Figure 17: Blood Sugar levels in response to glucose tolerance test at CPNA 28 days—solid pink lines represent term females (n=9), dashed pink lines represent preterm females (n=7), solid blue lines represent term males (n=10), and blue dashed lines represent preterm males (n=7). All data analysed using 2-way ANOVA and presented as group means ± SD.

Table 13: Average glucose tolerance test characteristics of term and preterm pups

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Term (n=10)</td>
<td>Preterm (n=7)</td>
</tr>
<tr>
<td>Baseline blood sugar level (mmol/L)</td>
<td>8 ± 1.1</td>
<td>7.1 ± 0.5</td>
</tr>
<tr>
<td>Absolute peak blood sugar level (mmol/L)</td>
<td>19 ± 2.5</td>
<td>18.3 ± 3.6</td>
</tr>
<tr>
<td>δ peak blood sugar level (mmol/L)</td>
<td>11 ± 1.8</td>
<td>11 ± 3.4</td>
</tr>
<tr>
<td>Area under curve</td>
<td>913 ± 145</td>
<td>988 ± 330</td>
</tr>
<tr>
<td># of individuals which didn’t return to base line after 180mins</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 13: Average glucose tolerance test characteristics of term and preterm pups - All data analysed using 2-way ANOVA and presented as mean ± SD.
3.4 Physiology at CPNA 28 days

Key organs in the biological pathway of NAFLD development - the liver, visceral fat and subcutaneous fat, were weighed and compared between treatment groups at the studies endpoint, CPNA day 28.

There was no effect of sex or prematurity on the weights of visceral and subcutaneous fat relative to body weight, consistent with our calculated measure of adiposity (PI) at CPNA day 28 (Figure 13c). On average, visceral fat deposition was consistent across all groups (prematurity p=0.9852, sex p=0.1069; term males, 0.2 ± 0.1%, vs. preterm males, 0.2 ± 0.1% vs. term females, 0.1 ± 0.07% vs. preterm females 0.2 ± 0.06%; Table 14) as were subcutaneous deposits (prematurity p= 0.5464, sex p=0.5145; term males, 0.8 ± 0.2%, vs. preterm males, 0.8 ± 0.2% vs. term females, 0.8 ± 0.1% vs. preterm females 0.8 ± 0.2%; Table 14).

Livers were significantly heavier (relative to body weight) in both male and female preterm animals (p=0.0449; term males, 3.3 ± 0.2%, vs. preterm males, 3.6 ± 0.2% and term females, 3.3 ± 0.3% vs. preterm females 3.7 ± 0.4%; Table 14). But were not different between males and females (p=0.9191).

3.4.1 Hepatic physiology

To determine if preterm livers deviated in other aspects of physiology, steatosis, inflammation and fibrosis of the liver was quantified and analysed at CPNA day 28.

Preterm birth had a significant effect on hepatic steatosis with higher NAS steatosis scores for male and female preterm pups than terms pups (p=0.0428; term females, 0.44 ± 0.53, vs. preterm females, 0.71 ± 0.49 and term males, 0.3 ± 0.48 vs. preterm males 0.75 ± 0.46; Figure 18a, Figure 19). Sex had no significant effect (p=0.7515).

The average number of inflammatory foci, viewable in a x100 magnification field of view (FOV) was not significantly different between preterm and term cohorts. The number of inflammatory foci was especially variable in the male preterm cohort (prematurity p=0.4823, sex p=0.4086; term females, 0.13 ± 0.26 foci/FOV vs. preterm females 0.07 ± 0.10 foci/FOV, and term males, 0.06 ± 0.09 foci/FOV, vs. preterm males, 0.05 ± 0.07 foci/FOV; Figure 12b, Figure 20).

Hepatocyte ballooning, an indication of advanced inflammation was not observed within any samples across all group, nor was portal, periportal or perisinusodial fibrosis (Figure 21).
Table 14: Organ weights relative to body weight of term and preterm pups at CPNA 28 days

<table>
<thead>
<tr>
<th></th>
<th>Male</th>
<th></th>
<th>Female</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Term (n=10)</td>
<td>Preterm (n=8)</td>
<td>Term (n=9)</td>
<td>Preterm (n=7)</td>
</tr>
<tr>
<td>Liver (% body weight)</td>
<td>3.3% ± 0.2%</td>
<td>3.6% ± 0.2%*</td>
<td>3.3% ± 0.3%</td>
<td>3.7% ± 0.4%*</td>
</tr>
<tr>
<td>Visceral Fat (% body weight)</td>
<td>0.2% ± 0.1%</td>
<td>0.2% ± 0.1%</td>
<td>0.1% ± 0.07%</td>
<td>0.2% ± 0.06%</td>
</tr>
<tr>
<td>Subcutaneous Fat (% bodyweight)</td>
<td>0.8% ± 0.2%</td>
<td>0.8% ± 0.2%</td>
<td>0.8% ± 0.1%</td>
<td>0.8% ± 0.2%</td>
</tr>
</tbody>
</table>

Table 14: Organ weights relative to body weight of term and preterm pups at CPNA 28 days - All data analysed using 2-way ANOVA and presented as mean ± SD. * denotes significance of p < 0.05.

Figure 18: Physiological characteristics of term and preterm liver tissue at CPNA 28 days. (A) Hepatic steatosis NAS (B) Hepatic inflammatory foci

Figure 18: Physiological characteristics of term and preterm liver tissue at CPNA 28 days. Solid pink bars represent term females (n=9), hashed pink bars represent preterm females at (n=7), solid blue bars represent term males (n=10), and blue hashed bars represent preterm males (n=8). (A) Average NAS steatosis score (0 - 3) at CPNA 28 days (B) Average number of inflammation foci / field of view at CPNA 28 days. All data analysed as 2-way ANOVA and presented as mean ± SD. * denotes significance of p < 0.05.
Figure 19: Examples of oil red o stained hepatic steatosis observed in terms and preterms.

- Lipid droplets (red) among hepatocytes (light blue) and hepatocyte nuclei (dark blue).

(A) Image from a test sample containing the least amount of steatosis observed. Lipid droplets covered 0.112% of total image area receiving a NAS steatosis score of 0.

(B) Image from a test sample representative of the average % steatosis coverage in term males. Lipid droplets covered 3.564% of total image area and was given a NAS steatosis score of 0.

(C) Image from a test sample representative of the average % steatosis coverage in preterm males. Lipid droplets covered 4.603% of total image area and was given a NAS steatosis score of 0.

(D) Image from a test sample containing the most amount of steatosis observed. Lipid droplets covered 13.23% of total image area receiving a NAS steatosis score of 1.

All images are of 12µm thick sections taken from frozen tissue, stained with oil red o and photographed at 200x magnification.
Figure 20: Examples of H&E stained hepatic inflammation and steatosis in terms and preterms

White blood cells (dark purple) among hepatocytes (pink) and hepatocyte nuclei (light purple). (A) Hepatic tissue free from inflammatory foci, photographed at x100 magnification. (B) Inflammatory focus (circled in black) within hepatic tissue, 100x magnification. (C) A large inflammatory focus (circled in black) within hepatic tissue, 100x magnification. Insert: Close up of the inflammatory focus at 200x magnification.

White blood cells comprising the focus are clearly visible (D) Steatotic hepatocytes characterised by round vacuoles (white) where lipids existed prior to tissue processing, 100x magnification Insert: A close up of the same steatotic tissue at 200x magnification.

All images are of 4µm thick sections taken from paraffin embedded tissue and stained with haematoxylin and eosin.
Figure 21: Examples of hepatic tissue stained with Masson’s trichrome

(A) (B)

(C) (D)

Figure 21: Examples of hepatic tissue stained with Masson’s trichrome. Collagen fibres (green) surrounding vascular and ductal lumen (white) among hepatocytes (pink). A, B, C, D) Images from test samples in which perisinusoidal, periportal or portal fibrosis was not observed. Vascular collagen surrounding portal triads is clearly stained green. All images are of 4µm thick sections taken from paraffin embedded tissue, stained with Masson’s trichrome and photographed at 200x magnification.
3.5 Hepatic molecular changes

3.5.1 Essential and conditionally essential amino acids

Preterm birth had a significant effect on hepatic arginine concentration, with lower levels in preterms compared to terms (p<0.001; term females, 0.024 ± 0.005nmol/mg, vs. preterm females, 0.016 ± 0.005nmol/mg, term males, 0.049 ± 0.019nmol/mg, vs. preterm males 0.016 ± 0.005nmol/mg; Figure 22a). Arginine concentration was also significantly affected by sex (p=0.0094) with greater levels of arginine in term males than term females. There was a significant interaction between sex and prematurity (p=0.0107). Both male and female preterms had lower levels of arginine than the terms, however the effect of prematurity was greater in the males. In addition, an inverse correlation in hepatic lipid accumulation and arginine was observed (r=-0.4835, p=0.0106; Figure 23a).

Hepatic concentration of histidine was not significantly affected by preterm birth or sex. Concentrations were similar across all groups (prematurity p=0.6025, sex p=0.8856; term females, 6.3 ± 1.3, preterm females, 6.5 ± 0.8nmol/mg, term males, 6.7 ± 1.3nmol/mg and preterm males 6.0 ± 0.9nmol/mg; Figure 22b). Histidine was not significantly correlated with hepatic steatosis (r=-0.3241, p=0.1140; Figure 23b).

Hepatic lysine concentration was not significantly affected by prematurity or sex, however preterm females had greater levels of lysine on average than term females (preterm females, 6.0 ± 1.3nmol/mg, vs. term females, 5.5 ± 1.2nmol/mg), and preterm males had lower levels of lysine on average than term males (term males, 6.6 ± 1.6nmol/mg and preterm males 5.4 ± 0.9nmol/mg.), p=0.4115 (Figure 22c), although there was no significant interaction between sex and prematurity (p=0.0687). Lysine was found to be inversely correlated with accumulation of hepatic lipids (r=-0.4584, p=0.0162; Figure 23c).

Concentration of methionine within the liver was not significantly affected by prematurity or sex, (prematurity p=0.0811, sex p=0.4664; term females, 1.0 ± 0.2nmol/mg vs. preterm females 0.8 ± 0.3nmol/mg, and term males, 0.9 ± 0.2nmol/mg, vs. preterm males, 0.8 ± 0.2nmol/mg; Figure 22d). Methionine was not correlated with hepatic steatosis (r=-0.0541, p=0.7929; Figure 23d).

Preterm birth significantly affected phenylalanine concentration, decreasing hepatic concentrations, especially in the male preterm cohort (prematurity p=0.0169, sex p=0.4538; term females, 0.8 ± 0.3nmol/mg vs. preterm females 0.7 ± 0.2nmol/mg, and term males, 0.9 ± 0.4nmol/mg, vs. preterm males, 0.4 ± 0.1nmol/mg; Figure 22e) There was no association found between phenylalanine concentration and hepatic deposition (r=-0.1090, p=0.5809; Figure 23e).

Hepatic concentration of threonine was not significantly affected by prematurity or sex (preterm females, 6.7 ± 1.1nmol/mg, vs. term females, 5.9 ± 2nmol/mg, and term males, 7.6 ± 1.9nmol/mg vs. preterm males
6.1 ± 1.3nmol/mg) (prematurity p=0.5808, sex p=0.3620; Figure 22f). This was not a significant interaction (p=0.0905). Threonine concentration was inversely correlated with hepatic lipid deposition (r=-0.5678, p=0.0031; Figure 23e).

Tryptophan in the liver was altered significantly by preterm birth with decreased concentration in both male and female preterm animals (p=0.0242; term females, 0.62 ± 0.16nmol/mg, vs. preterm females, 0.46 ± 0.07nmol/mg and term males, 0.69 ± 0.18nmol/mg vs. preterm males 0.53 ± 0.22nmol/mg; Figure 22g0). There was no correlation between tryptophan concentration and quantification of hepatic lipids (r=-0.1154, p=0.5511; Figure 23f).
Figure 22: The effect of preterm birth on essential amino acid concentration in the liver at CPNA 28 days

(A) Arginine

(B) Histidine

(C) Lysine

(D) Methionine
Figure 22: The effect of preterm birth on essential amino acid concentration in the liver at CPNA 28 days. Solid pink bars represent term females, hashed pink bars represent preterm females, solid blue bars represent term males, and blue hashed bars represent preterm males. (A) Arginine concentration – term females n=7, preterm females n=5, term males n=9, preterm males n=8. (B) Histidine concentration – term females n=7, preterm females n=6, term males n=7, preterm males n=6. (C) Lysine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=6. (D) Methionine concentration – term females n=5, preterm females n=6, term males n=8, preterm males n=8. (E) Phenylalanine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=7. (F) Threonine concentration – term females n=6, preterm females n=6, term males n=7, preterm males n=7. (G) Tryptophan concentration – term females n=8, preterm females n=7, term males n=8, preterm males n=8.

All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significant effect of prematurity, p < 0.01.
Figure 23: The correlation between hepatic lipid deposition and essential amino acid concentrations in the liver at CPNA 28 days

(A) Arginine

(B) Histidine

(C) Lysine

(D) Methionine
Figure 23: The correlation between hepatic lipid deposition and essential amino acid concentrations in the liver at CPNA 28 days. (A) Arginine (n=27). (B) Histidine (n=25) (C) Lysine (n=27) (D) Methionine (n=26) (E) Phenylalanine (n=28). (F) Threonine (n=25) (G) Tryptophan (n=29). All data analysed using Pearson correlation. * denotes significance of correlation, p < 0.05.
3.5.1.1 Branched chain amino acids

Preterm birth significantly decreased hepatic concentration of isoleucine in male and female preterm animals compared to terms (p = 0.0319; term females, 2.7 ± 0.9nmol/mg vs. preterm females 2.4 ± 0.4nmol/mg; and term males, 3.2 ± 1.1nmol/mg, vs. preterm males, 2.2 ± 0.4nmol/mg Figure 24a). Isoleucine was also inversely correlated with hepatic lipid accumulation (r=-0.6030, p=0.0009; Figure 25a).

Hepatic leucine concentration was significantly altered by prematurity, with depletion of leucine observed in the preterms (p=0.0174; term females, 6.0 ± 1.8nmol/mg, vs. preterm females, 5.1 ± 1.2nmol/mg and term males, 6.8 ± 1.9nmol/mg, vs. preterm males, 4.7 ± 0.8nmol/mg; Figure 24b). Leucine was inversely associated with hepatic lipid deposition (r=-0.5943, p=0.0011; Figure 25b).

As with the other branched chain amino acids (BCAAs), preterm birth significantly affected hepatic valine concentration with significantly lower levels of valine in preterms compared to terms (p=0.0365; term females, 4.4±1.2nmol/mg, vs. preterm females, 3.9 ± 0.4nmol/mg, and term males, 4.9 ± 1.5nmol/mg, vs. preterm males, 3.5 ± 0.7nmol/mg; Figure 24c). A moderate inverse association between hepatic lipid deposition and valine concentration was also observed (r=-0.6567, p=0.0002; Figure 25c).
Figure 24: The effect of preterm birth on branched chain amino acid concentrations in the liver at CPNA 28 days

(A) Isoleucine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=6. 
(B) Leucine concentration – term females n=9, preterm females n=8 term males n=7, preterm males n=8. 
(C) Valine concentration – term females n=8, females n=7, term males n=8, preterm males n=6. All data analysed as 2-way ANOVA and presented as group means ± SD. * denotes significant effect of prematurity, p < 0.01.
Figure 25: The correlation between hepatic lipid deposition and branched chain amino acid concentrations in the liver at CPNA 28 days.

(A) Isoleucine (n=27) (B) Leucine (n=27) (C) Valine (n=27). All data analysed using Pearson correlation. * denotes significance of correlation, p < 0.05.
3.5.2 Non-essential amino acids

Preterm birth had a significant effect on hepatic concentration of alanine with significantly lower levels of alanine in preterms animals compared to terms (p <0.001; term females, 25.9 ± 7.2nmol/mg, vs. preterm females, 17.71 ± 6.1nmol/mg, and term males, 32.4 ± 6.2nmol/mg, vs. preterm males, 19.0± 3.3nmol/mg; Figure 26a). Alanine concentration was not significantly altered by sex (p=0.0927). Hepatic lipid accumulation was not significantly correlated with alanine levels (r=-0.3388, p=0.0778; Figure 27a).

Citrulline concentration was not significantly affected by preterm birth or sex, (prematurity p=0.3114, sex p=0.3705; term females, 0.62 ± 0.22nmol/mg, vs. term males, 0.60 ± 0.25nmol/mg and preterm females, 0.63 ± 0.23nmol/mg and preterm males 0.63 ± 0.23nmol/mg; Figure 26b). There was no significant interaction between sex and prematurity (p=0.5914), and no association with hepatic lipid deposition (r=-0.0126, p=0.9483; Figure 27b).

Cysteine hepatic concentration was significantly altered by premature birth, with a large increases in concentration observed in male and female preterms (p<0.001; term females, 1.44 ± 1.44nmol/mg, vs. preterm females, 3.43 ± 0.98nmol/mg, and term males, 0.51 ± 0.22nmol/mg, vs. preterm males, 2.55 ± 0.76nmol/mg; Figure 26c). Cysteine concentration was also significantly altered by sex, with even greater increase seen in the females cohorts (p for sex =0.0191). Cysteine concentration was positively correlated with lipid accumulation within the liver (r=0.6152. p=0.0006; Figure 27c).

Preterm birth significantly depleted hepatic glycine concentration when compared to term cohorts (p=0.001; term females, 110.0 ± 19.9nmol/mg, vs. preterm females, 95.0 ± 8.9nmol/mg, term males, 134.4 ± 29.1%, vs. preterm males 91.1 ± 9.3nmol/mg; Figure 26d). Hepatic glycine concentration and lipid deposition were inversely associated (r=-0.6352, p=0.0004; Figure 27d).

Ornithine concentration in the liver was unaffected by prematurity or sex (prematurity p=0.7368, sex=0.0795; term females, 8.2 ± 3.5nmol/mg vs. term males, 11.4 ± 2.8nmol/mg, preterm females, 9.2 ± 1.6nmol/mg vs. preterm males 9.7 ± 2.4nmol/mg; Figure 26e). A correlation between hepatic lipid accumulation and ornithine was not observed (r=-0.0396, p=0.8414; Figure 27e).

Preterm birth and sex both significantly affected hepatic proline concentration with lower levels of proline in both preterm and female cohorts compared to the male term cohort (prematurity p=0.0220, sex p=0.0311; term females, 8.6 ± 1.6nmol/mg, vs. preterm females, 8.1 ± 1.3nmol/mg, and term males, 13.81 ± 4.8nmol/mg, vs. preterm males, 8.4 ± 2.1nmol/mg; Figure 26f). Additionally, proline was inversely associated with hepatic lipid accumulation (r=-0.4917, p=0.0107; Figure 27f).

Hepatic serine concentration was significantly altered by prematurity with lower serine levels in preterm cohorts (p=0.0100; term females, 29.1 ± 9.1nmol/mg vs. preterm females, 25.1 ± 3.1nmol/mg and, term
males, 36.7 ± 9.7nmol/mg vs. preterm males, 25.4 ± 2.4nmol/mg; Figure 26g). Lipid deposition within the liver was inversely associated with serine levels (r=-0.6823, p=0.0001; Figure 27g).

Prematurity had a significant effect on tyrosine concentration in the liver with significantly lower levels of tyrosine in preterm animals (p=0.0009; term females, 3.5 ± 1.1nmol/mg, vs. preterm females, 2.6 ± 0.5nmol/mg, and term males, 3.8 ± 1.0nmol/mg, vs. preterm males, 2.4 ± 0.3nmol/mg; Figure 26h). A correlation between hepatic tyrosine and lipid accumulation was also observed (r=-0.6121, p=0.0007; Figure 27h).
Figure 26: The effect of preterm birth on Non-essential amino acid concentrations in the liver at CPNA 28 days.

(A) Alanine

(B) Citrulline

(C) Cysteine

(D) Glycine

*P < 0.05
Figure 26: The effect of preterm birth on Non-essential amino acid concentrations in the liver at CPNA 28 days. Solid pink bars represent term females, hashed pink bars represent preterm females, solid blue bars represent term males, and blue hashed bars represent preterm males. (A) Alanine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=7. (B) Citrulline concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=8. (C) Cysteine concentration – term females n=7, preterm females n=6, term males n=8, preterm males n=7. (D) Glycine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=7. (E) Ornithine concentration – term females n=7, preterm females n=6, term males n=8, preterm males n=8. (F) Proline concentration – term females n=6, term males n=8. (G) Serine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=6. (H) Tyrosine concentration – term females n=8, preterm females n=6, term males n=8, preterm males n=6. All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significant effect of prematurity, p<0.01.
Figure 27: The correlation between hepatic lipid deposition and non-essential amino acid concentrations in the liver at CPNA 28 days.

(A) Alanine

(B) Citrulline

(C) Cysteine

(D) Glycine
Figure 27: The correlation between hepatic lipid deposition and non-essential amino acid concentrations in the liver at CPNA 28 days. (A) Alanine (n=28) (B) Citrulline (n=29) (C) Cysteine (n=27) (D) Glycine (n=27) (E) Ornithine (n=28) (F) Proline (n=26) (G) Serine (n=27) (H) Tyrosine (n=27). All data analysed using Pearson correlation. * denotes significance of correlation, p < 0.05.
3.5.3 Amino acid profiles of male and female preterms

Figure 28: Total amino acids significantly altered with preterm birth

Total amino acids significantly altered with preterm birth

![Diagram showing amino acids altered with preterm birth]

- **6 Unchanged**
- **1 Changed in males**
- **11 Changed in both males and females**

**Total amino acids measured = 18**

Figure 28: Total amino acids significantly altered with preterm birth – grey segment represents amino acids not significantly altered by preterm birth, blue segment represents amino acids significantly altered in preterm males only, purple segment represents amino acids significantly altered in both male and female preterm animals.
Figure 29: Preterm amino acid concentrations - Baseline represents mean concentration of amino acids in preterm pups. Solid pink bars represent preterm females, solid blue bars represent preterm males. All data expressed as mean percentage change. * denotes significance of p < 0.01.
4 Discussion

4.1 The preterm phenotype

4.1.1 Growth

Our study found significant alterations in preterm growth, deviating from the term phenotype (Figure 30). Many of these trajectories observed in our study are consistent with human preterm observations, however some key differences in adipose tissue development was observed.

At birth our preterm cohort was significantly lighter and shorter than term born counterparts, as observed in human preterm birth. By TEA, weight and length had caught up to that of the terms, suggesting that growth of the preterms was comparable to foetal growth of the terms. However, on closer examination of growth during the preterm period, differences were observed in weight loss of our terms and preterms. Immediately after birth, preterms experienced a period of weight loss significantly greater in severity than the terms. This has also been observed in premature human birth and can lead to declining health without intervention. Weight gain and linear growth rates of the preterm pups were consistent overall, however deviations from the term phenotype were observed, including; reduced weight gain in preterm females 2 weeks after TEA until the studies endpoint; and limited linear growth (CRL) of preterm females (TEA – CPNA 21), and preterm males (CPNA 14 – CPNA 28). These findings are comparable to ex-preterm growth trajectory in humans, who were found to remain smaller than their term counterparts until at least 18 years of age.

The finding that our preterms displayed greater adiposity at birth than terms was surprising as adipocyte development has previously been described as occurring late in the 3rd trimester. Birth adiposity in both terms and preterms was highly variable, and maybe indicative of confounding variation in maternal and litter characteristics. In order to minimise the impact of litter and maternal effects, REML analysis and nested two-way ANOVAs should be performed, however this would require a cohort much larger than the current study. Throughout the study period, adiposity as assessed by ponderal index was highly variable in all cohorts. Overall, female preterms significantly diverged from term females in their adiposity development, however by the studies endpoint their adiposity was not significantly different from the other cohorts. Preterm males had extended periods of divergence from term males, however adiposity development was not significantly altered overall. Increased adiposity, a common complication in adult ex-preterms which contributes to metabolic syndrome, was not observed in our relatively young pups, however increased adiposity development during later life (as occurs in humans) cannot be ruled out. Further research tracking adiposity into later life stages is required to definitively draw such conclusions.
We were unable to determine if accelerated catch up growth, a common feature in preterm human growth trajectory, was a factor within our preterm cohort. Comparisons between FWG of the terms and preterms were made during the first week of life, however this is not a valid comparison. To determine if accelerated catch up growth was a factor in our cohort, a foetal ontogeny study is required to determine ‘normal’ foetal growth rates between GA 62 and 69 days. In humans accelerated catch up growth is likely due to clinical practice, where accelerated growth has historically been promoted to mitigate the effects (mortality and decreased cognitive function) of low birth weight. This practice is currently debated as growth acceleration, or ‘catch up growth’ has been liked to adverse metabolic and cardiovascular conditions development later in life.

While our preterms experienced a period of initial instability, growth from TEA onwards was consistent with term trajectories. Males tended to be of greater weight throughout the study period, displaying the sexual dimorphism in growth that is expected in both guinea pigs and humans.

**Key Findings:**

- Postnatal weight loss and growth trajectory of our preterm cohorts was consistent with human preterm growth.
- By TEA, preterms had caught up to terms in weight and length, although preterm growth rates slowed after this catch up period.
- Adiposity was highly variable in both term and preterm cohorts, increased adiposity associated with metabolic syndrome had not yet developed in our young cohort.

### 4.1.2 Metabolism

Key metabolic alterations in the preterm phenotype were observed within our study. Initial fluctuations in our preterms fasted BSLs could result in alterations in NAFLD associated pathways, predisposing the preterm to NAFLD development later in life (Figure 30). Preterm animals had significantly lower fasted BSL at birth than terms, consistent with observations of increased hypoglycaemia in human preterm and LBW neonates. Hypoglycaemia in the neonate indicates dysfunction in metabolic regulatory pathways, with an imbalance in use and supply of glucose. This is possibly due to immaturity of endocrine and enzymatic controls in the preterm and/or lack of glycogen and fat stores which develop primarily during the 3rd trimester. Without intervention, hypoglycaemia can result in severe neurological and growth deficits. Upon reaching TEA, our preterm cohorts’ fasted BSL had risen to be significantly higher than the terms, a commonly described postnatal condition in preterm humans. Postnatal hyperglycaemia may occur.
through immaturity of the pancreatic β cells and resistance to the insulin they produce. This pattern of hyperglycaemia following hypoglycaemia has previously been described in preterm human infants. We observed deviations in overall BSL trajectory in both male and female cohorts throughout the study period, indicating altered metabolism within the preterm cohort which may have altered downstream pathways associated with NAFLD. Harader et al. demonstrated that hyperglycaemia exacerbated early phase NALFD development in mice through increased inflammatory cytokine release. Hyperglycaemia is also associated with insulin resistance which increases FFA by encouraging adipose tissue lipolysis and de novo lipogenesis within the liver. This excess in FFA is stored in the liver, resulting in increased steatosis, a hallmark of NAFLD.

Glucose tolerance tests (GTTs) were administered at 28 days to give a clearer indication of metabolic function and insulin resistance in ‘early childhood’. No significant differences were found, indicating stabilisation of glucose homeostasis and insulin resistance (although insulin levels throughout life and in response to the oral GTT have yet to be assessed, and may offer some additional insight into metabolic function). In future, GTTs could be administered prior to ‘early childhood’ to better establish if insulin resistance is occurring in the preterms prior to BSL stabilisation.

Limited circulating blood volume prevented administration of GTTs to the guinea pigs at multiple developmental time points. Preliminary data gathered by our group from a cohort of 6 month old term and preterm guinea pigs showed an effect of prematurity (p=0.02; See appendix 7.2, Page 98). This implies metabolic function of the preterm may fluctuate during development, with insulin resistance developing later in life. Potentially, metabolic function of our preterm cohort may have diverged from the terms in later life (after our study end point).

**Key Findings:**

- At birth, low fasting BSL in preterms is likely due to immaturity in endocrine and enzymatic metabolic controls and underdeveloped glycogen and fat storage.
- By TEA, fasted BSL of the preterm has risen significantly above the terms indicating metabolic dysfunction and possible insulin resistance.
- Dysrupted metabolic function may have altered downstream pathways associated with NAFLD development.
4.1.3 Hepatic physiology

The steatosis and inflammation observed in our preterm cohort was not severe enough for any of our subjects to be diagnosed with NAFLD in the current study. However, significant increase in steatosis observed in our preterm cohort can be considered a precursor marker of potential NAFLD development.

Isolated steatosis is the first histologically observable hepatic alteration in the development of NAFLD. Increased steatosis could be the outcome of dysfunction in one of 4 mechanisms: FFA uptake, de novo lipogenesis, lipid oxidation or lipid exportation from the liver. Increased FFA uptake is implicated in our study, as fluctuating fasting BSL in our preterms may indicate insulin resistance with adipose tissue lipolysis and de novo lipogenesis as a result (Figure 30).

Preterms also exhibited a slight increase in inflammatory foci, although due to variation within the groups, this was not a significant result. Hepatic inflammation can be caused by systemic (originating from adipose tissue dysfunction), or local inflammation. Hepatic steatosis occurs in an environment of excess lipid intermediates. These intermediates have been found to place strain on mitochondria and endoplasmic function resulting in cytokine release and ROS production. The significant but relatively low level of steatosis observed in our preterm cohort, may not yet be at a level in which cellular dysfunction occurs, as reflected by our inflammation findings. Fibrosis was not observed in any of the samples collected. This is unsurprising as onset occurs in response hepatocyte apoptosis resulting from steatosis and inflammation.

Our finding of increased hepatic steatosis, although relatively small, is significant especially as the pups are of a relatively young age. Previous human preterm studies have found increased rates of steatosis and liver damage in comparably older (young adult) patients. Further studies are required to determine if the increased steatosis we found during ‘early childhood’ would continue to develop, stabilise or ameliorate with age. Likewise, inflammation may increase with age, and fibrosis may develop later in life.

Key Findings:

- NAFLD was not present during ‘early childhood’ in any of our subjects, however steatosis, a precursor to its development was.
- Significant increase of steatosis in the preterms could be an effect of increased FFA uptake in the liver.
4.1.4 Hepatic molecular alterations

Amino acids can be categorised as essential or non-essential, dependant on the body’s ability to synthesise the amino acids from precursor molecules. Essential amino acids must be obtained from dietary sources. Conditionally essential acids, ordinarily biosynthesised, may require dietary supplementation during periods of physiological stress, such as premature birth. Amino acid deficiency or excess and can result in a broad range of pathologies in all aspects of physiological function. Depletions may be caused by biosynthesis dysfunction or insufficient intake, while excess accumulation may be the result of an inability to break down or convert the molecule.

Similarly to earlier studies, we observed amino acid depletions associated with liver damage and lipid deposition, and an increased concentration of cysteine which inhibits lipid clearance from the liver, in our preterm offspring (Figure 30).

4.1.4.1 Cysteine, serine, glycine, and threonine

Increased levels of cystine (the more stable, oxidised dimer form of cysteine) have been observed in plasma and urine of patients with liver damage. Walshe et al found increased excretion of cystine was directly related to increased fasting plasma levels of cystine suggesting impairment in amino acid metabolism associated with liver damage. Excessive dietary intake of cysteine was found to promote fatty liver, while methionine (not significantly altered in our study) and choline were preventative. Cysteine, dramatically increased in the preterm cohorts of our study, is a conditional amino acid. It has been suggested that cysteine is not synthesised in preterms, however Riedijk et al found no evidence of this in their human preterm cohort. Furthermore, a downstream metabolite of cysteine, hydrogen sulphide, has been found to be raised in preterm newborns. A pathway proposed by Toohey et al suggests excessive cystine oxygenation results in sulfur sulfane (S°) - binding intermediates which deplete free S°, preventing lipoprotein export from the liver, suggesting a key role in hepatic lipid accumulation (steatosis) and NAFLD development. Conversely, methionine is thought to increase available S° therefore acting as a lipotropic agent. Cysteine is further implicated as a contributing factor to the NAFLD predisposition observed in our preterms, by the positive correlation we observed between hepatic lipid accumulation and hepatic cysteine concentration. Further research is required to determine the mechanism by which cysteine is increased in the preterms and the role it plays in hepatic lipid deposition.

Serine, a non-essential amino acid is a precursor molecule of cysteine and was depleted in preterms of the current study. Supplementation of L-serine has resulted in reduced hepatic lipid accumulation in rats, which was described as a potential result of accelerated homocysteine metabolism. Glycine and cysteine share serine as a key precursor to their biosynthesis. The increased cysteine observed in our preterms may
therefore be a contributing factor in the preterms glycine deficiency, as the serine ordinarily used in glycine production could have been seconded to the cysteine synthesis pathway. Threonine, an essential amino acid required for the biosynthesis of serine and glycine, was not altered within our preterm cohort, however lower levels were moderately correlated with the increased hepatic steatosis observed in our study. This correlation could simply be a reflection of threonine’s role in cysteine and serine synthesis, which are directly associated with hepatic lipid accumulation. As threonine is an essential amino acid, it maybe the limiting factor when dietary intake cannot keep up with the demand increased cysteine synthesis imposes, resulting in reduced serine and glycine levels.

4.1.4.2 Leucine, isoleucine, valine and alanine

Serum levels of branched chain amino acids (BCAAs) have been found to be decreased with liver damage, and supplementation of BCAAs in rats with chronic liver damage showed reduction in hepatic apoptosis. Additionally, patients with chronic liver disease receiving BCAA supplementation had improved insulin response and beta cell function, indicating a potential role of BCAAs in insulin homeostasis. This protective function of BCAAs may partly be due to their ability to induce activation of genes involved in ROS inhibition and hepatic oxidation repair. The three essential BCAAs—leucine, isoleucine and valine, were all significantly depleted in our preterm cohorts, furthermore their depletions were moderately correlated with increased hepatic steatosis. It is unclear if BCAA depletion is a result or outcome of liver damage, however preterms have been identified as being at risk of receiving inadequate levels of BCAAs for two potential reasons: inadequate nutrition reserves due to shortened gestation, and lowered ability to absorb nutrients due to an underdeveloped gastrointestinal tract.

Furthermore, alanine, significantly reduced in our studies preterms, is synthesised in the body from pyruvate and BCAAs. Potentially the depletion of valine, leucine and isoleucine observed in the male preterms of our study has prevented adequate production of alanine. As with the BCAAs, alanine was found to reduce liver damage in rats.

4.1.4.3 Tryptophan

Tryptophan, an essential amino acid with antioxidant properties and sourced from maternal milk was depleted in our preterm cohorts. In 2014 two studies were published highlighting tryptophan as a potential treatment option for NALFD. Ritze et al demonstrated NAFLD attenuation in mice receiving tryptophan orally; and Celinski et al observed lowered levels of hepatic triglyceride and inflammatory cytokines within NAFLD patients receiving tryptophan and melatonin supplementation. Potentially, remedying the tryptophan deficit observed in the preterms of the current study could have a similar ameliorative effect on the increased steatosis we observed.
4.1.4.4 Arginine and proline

Arginine, found to be depleted only in preterm males of the current study has been described as a conditionally essential amino acid in preterms. This discrepancy among male and female preterms observed in the current study may indicate a sexual dimorphism in the ability to self-synthesise arginine while under stress, with males having less success. This is contrary to a study by Ruzafa et al, where male mice had higher concentrations of arginine in muscle, plasma and kidney tissue when dietary arginine was restricted. Unfortunately, Ruzafa’s study was not designed to reflect arginine concentration when under physiological stress, so the sexual dimorphism they observed may not translate to the preterm phenotype, although it is interesting to note differing capabilities of arginine synthesis between sexes exists. Arginine supplementation has been found to increase hepatic blood flow and decrease steatosis in rats.

Proline, reduced in our preterm cohorts, was shown by Tomlinson et al to be the key precursor molecule for arginine synthesis in preterm neonatal pigs and humans. Therefore reduced proline may contribute to the preterm males lowered ability to synthesise arginine. Tomlinson et al’s study also highlights alternate pathways of arginine synthesis. Potentially males and females have differing ability to use these pathways, which could contribute to the sexual dimorphism in arginine concentration observed in our study, however further research is needed to come to such a conclusion.

4.1.4.5 Tyrosine and phenylalanine

Tyrosine, an essential amino acid, was depleted in our preterm males and was moderately inversely correlated with hepatic lipid accumulation. Phenylalanine, a non-essential amino acid synthesised in the body from phenylalanine was similarly depleted in preterm males, although not correlated with steatosis.

Key Findings:

- Increased cysteine may inhibit lipid exportation from the liver, resulting in hepatic steatosis.
- BCAA depletion is associated with liver damage and oxidation. Supplementation with BCAAs ameliorates liver damage.
- Many of the amino acids found depleted in our study reduced hepatic lipid deposition when administered in previous studies.
Figure 30: Proposed biological pathway of NAFLD development in the preterm

Alterations to the preterm phenotype observed in the current study identified in green. In blue, the amino acids found to be significantly altered in our preterm cohorts, alongside the biological processes they are associated with.
4.2 Strengths of the model

4.2.1 The guinea pig as a model of premature birth

The suitability of animal models varies depending on the mechanisms, anatomy and responses the study wishes to manipulate and observe. Rodent models are well established, but key differences in gestational development may lead to inaccurate translation when modelling premature birth. The use of sheep, although well described as a model of prematurity, are divergent from humans in their placentation.

Guinea pigs share similarities in antenatal development, parturition and placentation with humans. Development of the premature guinea pig paradigm, has enabled modelling of long term health outcomes of premature birth. Previous studies employed caesarean section, requiring the termination of the mother and eliminating the possibility for maternal-infant interaction. Berry et al’s innovation of medically induced labour, maintains maternal stimulation of the offsprings’ sympathetic adrenergic systems preventing variation in cardiometabolic function resulting from a lack of maternal care. Neonatal nutritional intake is also more accurately represented as the offspring are able to suckle with postnatal survival of the mother.

4.2.2 The guinea pig as a model of hepatic function

Guinea pigs have previously been established as a suitable model for studying hepatic function and lipid metabolism in the human due to key similarities in hepatic enzyme activity, lipoprotein transport, synthesis and storage, and response to dietary intervention. Guinea pigs have also been assessed on their ability to mimic human inflammation (a key component in NAFLD), and atherosclerosis (a potential outcome of NAFLD). Fernandez & Volek demonstrated the ability of guinea pigs to develop atherosclerosis when fed a high cholesterol diet and validated guinea pigs as a model to study inflammatory response during atherosclerosis development.

Currently there is no validation of a NAS specific to guinea pig presentation. In 2014 Laing et al set out to validate Kleiner et al’s NAS system for the use rodent trials. Comparing human and murine liver biopsies, discrepancies in the manifestation of NAFLD were observed, such as the relative rarity of hepatocellular ballooning within murine samples. As the guinea pig maybe a closer representation of the human hepatic system than the murine model, our quantification took hepatocellular ballooning into consideration. Hepatocellular ballooning was not observed in the current study, however this may be due to the limited severity of NAFLD within our cohort rather than its absence in the guinea pig model. Validation of a guinea pig NAS system using a spectrum of NAFLD severity would be worthwhile.
4.3 Limitations and considerations

4.3.1 Use of corticosteroids prior to preterm birth

The introduction of corticosteroids into the premature birthing paradigm may have introduced additional confounding variables as our term cohort was not exposed to antenatal corticosteroid treatment. However, the incorporation of such pharmaceutical intervention is imperative to the paradigm as offspring would not survive preterm delivery at this gestational age without it (Dyson et al, unpublished data).

Betamethasone, administered to encourage lung maturation in the preterm, is classed as a glucocorticoid due to its role in glucose metabolism. Betamethasone has been found to be associated with neonatal hypoglycaemia 152, which may account for the lowered birth BSL observed within our preterm cohort. As a group, glucocorticoids act on the liver and adipose tissue to regulate glucose homeostasis 153. Glucocorticoids have also been found to effect pathways key in the development of NAFLD; increasing lipolysis within adipose tissue, and stimulation of hepatic gluconeogenesis 154-156. It is not yet known if administration of betamethasone alone effects these pathways, however this research implicates betamethasone as a potential contributing variable to the increased steatosis observed within our preterm cohort.

Betamethasone is routinely administered in cases where there is a risk of preterm birth, so its use in our paradigm is analogous to the clinical setting 143,157. The ability to elucidate if NAFLD development is the result of preterm birth or the clinical treatment administered in preterm cases is an important distinction, therefore our research group currently has a study underway which aims to elucidate potential alterations betamethasone may have on guinea pig growth and metabolism. Understanding the effect of corticosteroids, including betamethasone will allow us to account for potential treatment variation in future studies.

4.3.2 Use of anaesthetics to euthanize

Pentobarbital, medetomidine and ketamine are routinely used for anesthetisation and euthanasia in animal studies, however may alter hepatic chemistry due to the livers role in metabolising these drugs. Overmyer et al observed changes in key liver metabolomics, especially with ketamine use and therefore recommended the use of isoflourane for anesthetisation 158. However, in the metabolomics observed in this study (amino acids) Overmyer et al found no significant alterations. Going forward, incorporation of Overeyer et al’s mammalian tissue collection strategy should be considered, especially when targeting molecular pathways found to have been altered by drug metabolism.
4.3.3 Lowered resolution of the glucose tolerance test

Due to ethical concerns, the GTT administered to our guinea pig cohort consisted of limited time points. Increased frequency of blood sugar readings was not possible due to the low circulating blood volume in the young guinea pigs (6.7ml/100g body weight). This resulted in lowered resolution of the glucose response curve, preventing in-depth analysis of the subject’s metabolic function (for example determination of glucose response phases). We were unable to find significant differences in the limited curve characteristics we were able to explore (peak BSL, Δ-peak BSL and AUC), however this may be a reflection of the tests lowered resolution rather than a similarity of metabolic response between terms and preterms.

Furthermore, development of a guinea pig insulin assay is currently underway by our colleague, Professor David Kennaway at the University of Adelaide. The ability to determine levels of circulating insulin in the guinea pig will provide a clearer demonstration of potential metabolism deviations in our preterm cohort, however validation of this assay was not completed during the timeframe of the current project.

4.3.4 Histological techniques

4.3.4.1 Cryosections and the development of ice artefact

As lipid are removed by the alcohol and xylol used in tissue paraffin processing, oil red o staining and analysis must be performed on frozen, cryosectioned tissue. This technique is associated with increased artefact deposition which may potentially impact on diagnosis. A technique championed by Tracy et al in 2002 gives an alternative to frozen tissue processing by describing a paraffin sectioning technique which ensures lipid fixation in formalin fixed tissue. The technique uses a process which enriches the tissue with additional lipids specifically binding to the naturally deposited lipids within the sample. Exposure to chromic acid then fixes the lipids and allows for the introduction of fat soluble stains.

The Tracy et al method was attempted for use on formalin fixed tissues collected for our study, however an adequate result was not attained (See 7.5 Appendix: Lipid fixation protocol validation, Page 102).

As the protocol was unable to be validated, frozen samples were used for oil red o analysis. To limit ice artefact development, tissue was snap frozen in liquid nitrogen and care was taken to avoid unnecessary temperature fluctuations when processing the tissue. The resulting slides appeared relatively free from ice artefact as tissue architecture appeared undisrupted.
4.3.4.2 Application of NAS to pre-NAFLD liver tissue

As our study was interested in the precursor state of NAFLD development, liver physiology was observed at a relatively young age. As a result, the characteristics associated with NAFLD (steatosis, inflammation and fibrosis) were minimal. The NAS was designed to stage progression of NAFLD, rather than determine a predisposition, and therefore, the scoring system is weighted to differentiate cases of greater severity than observed in our samples. To increase the detection of inflammation, the magnification at which hepatic H&E stained tissue was analysed was altered from Kleiner et al's' recommended x200 magnification to x100 magnification (used in Liang’s rodent NAS validation) 44,95. In doing so, a greater area of tissue was viewed, increasing the likelihood of observing inflammation foci. As the number of foci observed remained minimal even with decreased magnification, the average numbers of foci observed were statistically analysed rather than the inflammatory NAS score. This allowed differentiation between the majority of samples, which would have scored a ‘1’ in NAFLD activity. For future studies interested in liver physiology associated with NAFLD predisposition, validation of a NAS with increased sensitivity, through assessing the average number of foci over a greater sample area, would be a worthwhile endeavour.

4.3.4.3 Zonation of steatosis

Metabolic zonation within the liver has previously been described due to differential metabolic capabilities of hepatocytes relative to periportal proximity 161,162. This pattern is suspected in hepatic triglyceride accumulation 163. We therefore employed a method of sampling full face tissue sections through the lobe, providing a greater region of tissue to analyse than a biopsy would provide. By averaging steatosis and inflammation quantification across multiple sites of the sample, the possible effect of zonation is minimised. In order to maintain consistency, the lower right lobe of all subjects was assessed for steatosis. Future studies may look to assess steatosis in multiple lobes to ensure steatosis throughout the entire liver is reflected and enable greater accuracy of findings.

4.3.5 Differential NAFLD presentation in paediatric cases

Paediatric NAFLD presentation has been shown to differ from adult presentation 96,164. Suspecting NAFLD presented differently in paediatric and adult cases, Schwimmer et al set out to categorise manifestations of NAFLD in paediatric patients 96. They were able to class NASH presentation into 2 subtypes; Type 1 - steatosis with hepatocellular ballooning (typical of adult cases), and Type 2 - steatosis with portal inflammation/fibrosis without hepatocellular ballooning. Differences in the steatosis, inflammation and fibrosis of less advanced NAFLD cases were not investigated. As hepatic physiology was assessed at a young age in the current study, it could be argued that incorporation of Alkhouri et al’s Pediatric NAFLD Histological Score (PNHS) could more accurately quantify NAFLD manifestation in our study. The PNHS
recognises the importance of portal inflammation in severe cases of paediatric NASH. As NAFLD manifestation was not expected to be severe in our cohort, a NAS which incorporated rodent validation and with greater sensitivity was prioritised over use of the PNHS. Images from the current study have been retained and will be re-analysed using the PNHS to assess whether this is a more valid approach in the current context.

4.4 Potential future research

Characterising liver dysfunction and potential predisposition to NAFLD in our guinea pig model of prematurity provides a vehicle for studies where human trials are not possible. Results and potential interventions highlighted by the current study may then be translated into a clinical setting. In order to elucidate the mechanisms underpinning the ex-preterms’ predisposition to liver dysfunction and NAFLD development, it will be important to determine correlations between the metabolites we found to be altered in preterms, with genes and NALFD pathway function through targeted RNA-seq and principal component analysis (PCA). In the present study we have investigated early development of NAFLD precursors in the ex-preterm. Future studies will need to investigate if the markers of NAFLD predisposition found in our study results in increased NALFD severity later in life, or earlier presentation in ex-preterms relative to terms. In testing a cohort of guinea pigs treated in the same manner as this study but with an end point relative to human young adulthood (6 months CPNA in our cohort), we would be able to determine if the steatosis developed in our ‘young adult’ cohort increases, ameliorates or is stable with time. Such an approach would also allow for the testing of novel therapeutics in this vulnerable population.

In this study, many of the amino acids found to be depleted in the preterm phenotype have previously been described as altering liver function in rats. Correcting this deficit via maternal or postnatal supplementation regimes represents one such therapeutic strategy. Research into the effects and viability of supplementation of these amino acids maybe worthwhile as they may prove useful as interventional treatment in cases of preterm predisposition to metabolic dysfunction.

In humans it is clear that development of disease is multifactorial. The “two-hit” hypothesis states that the ex-preterm child/adult is vulnerable to a second insult such as a sedentary lifestyle or western diet. High fructose intake, common in the western diet has been proposed as a contributing factor to NAFLD development. Without intervention, preterm children are likely to be exposed to this diet which may act as a second ‘hit’, exacerbating predisposition to NALFD in preterms. By incorporating high fructose diet as a 2nd factor in this studies design, the interaction between diet and prematurity may also be elucidated.
5 Conclusion

This study has characterised the early childhood stage of the preterm phenotype by demonstrating preterm deviation from their full term counterparts in growth, metabolism, hepatic physiology and molecular alterations associated with NAFLD development. Hepatic steatosis and inflammation observed in our preterm cohorts were not severe enough for any of our relatively young subjects to be diagnosed with NAFLD. However, the significant increase in steatosis we observed in our preterms can be considered a precursor marker of potential NAFLD manifestation.

Our study was able to demonstrate that the developmental environment of prematurity results in significant changes to growth and metabolic function in the neonate. The majority of this deficit is reduced upon reaching term equivalent age, however, molecular and physiological changes within the liver persist into early childhood. By identifying metabolic, molecular and physiological alterations in the preterm guinea pig this study has taken the first steps in understanding the pathways involved in preterm susceptibility to NAFLD.

Furthermore, in identifying the preterms’ predisposition to NAFLD; the hepatic manifestation of metabolic syndrome, new opportunities are provided which may allow for early detection of metabolic syndrome susceptibility. Metabolic syndrome, a broader metabolic complication associated with increased mortality is known to affect ex-preterms at a relatively young age, decreasing their quality of life and inflating the burden on the healthcare system. By recognising the precursor markers of NAFLD at a young age, early intervention can be administered to attenuate the manifestation of NAFLD and metabolic syndrome.

Further research is required to determine the specific molecular pathways involved in NAFLD predisposition and potential future treatment options. Amino acids associated with liver damage that were found to be altered in our preterms, may provide a promising route for such studies.
6 Reference list


56. Cusi, K. Role of Insulin Resistance and Lipotoxicity in Non-Alcoholic Steatohepatitis. *Clinics in Liver*


95. Liang, W. *et al.* Establishment of a general NAFLD scoring system for rodent models and


7 Appendix

7.1 Biometric measurements

Figure 31: Twice weekly biometric measurements

(A) Hock-toe: measured from tip of hock to tip of middle toe (excluding claw).

(B) Hind limb: measured from ‘knee’ to hock.

(C) Crown-rump length: measured from crown where top of the ears attach to tailbone.
7.2 Preliminary data comparing GTT’s of 6 month old terms and preterms

Figure 32: Preliminary data comparing GTT results of 6 month old terms and preterms

6 month GTT - Area Under Curve

![Graph showing GTT results for female and male terms and preterms.]

Figure 32: Preliminary data comparing GTT results of 6 month old terms and preterms. Solid pink bars represent term females (n=7), hashed pink bars represent preterm females (n=8), solid blue bars represent term males (n=8), and blue hashed bars represent preterm males (n=7). All data analysed using 2-way ANOVA and presented as group means ± SD. * denotes significance of p < 0.05
7.3 Liver dissection and sampling

Figure 33: Liver dissection and sampling

(A) Excised liver, upper right lobe (1) collected as frozen sample for histology. Left lower lobe (2) collected as formalin fixed sample for histology. Remainder of liver (excluding gallbladder) was diced and sampled from multiple locations (3) and frozen for LC-MS/MS. (B) 5µm cross sections were taken from the widest part of the lobe in both frozen and fixed liver samples. (C) 5 fields of view were sampled from each section. Indicates the general areas sampled (not to scale).
7.4 Solutions

7.4.1 Phosphate Buffer

Disodium 23.7g
Sodium dihydrogen 6.75g
DH₂O 2L

7.4.2 Haematoxylin and Eosin

90% Ethanol

99% Ethanol 900ml
DH₂O 100ml

80% Ethanol

99% Ethanol 800ml
DH₂O 200ml

70% Ethanol

99% Ethanol 700ml
DH₂O 300ml

Acid Alcohol

99% Ethanol 1400ml
Tap Water 600ml
Concentrated HCl 20ml

Eosin

Stock solution

Eosin yellowish 1B423 20g
Ethanol 1600ml
DH₂O 400ml

Working solution

Stock solution 400ml
Ethanol 1200ml
Glacial acetic acid 8ml
DH₂O 240ml

Haematoxylin

Solution A

Haematoxylin 10g
70% Ethanol 150ml

Solution B

Aluminium potassium sulphate 200g
DH₂O 2000ml

Working solution

Solution A 150ml
Solution B 2000ml
Sodium iodate 1g
Filter

7.4.3 Masson’s Trichrome

Bouins solution

Picric acid (saturated) 75ml
Formaldehyde (37%) 25ml
Glacial acetic acid 5ml

Weigert’s Iron Haemotoxylin

Stock solution A

Hematoxylin 1g
95% Ethanol 100ml

Stock solution B

29% Ferric chloride in H₂O 4ml
DH₂O 95ml
Concentrated HCL 1ml

Working solution

Weigerts stock A 100ml
Weigerts stock B 100ml
Biebrich scarlet-acid fuchsin
1% Biebrich scarlet  90ml
1% Acid fushcin  10ml
Glacial acetic acid  1ml

Phosphomolybdic-phosphotungstic acid
5% phosphomolybdic-  25ml
5% phosphotungstic  25ml

Aniline Blue
Aniline Blue  2.5g
Glacial acetic acid  2ml
DH$_2$O  99ml

1% Acetic acid
Glacial acetic acid  1ml
DH$_2$O  99ml

Light green
Light green  1g
Acetic acid  1ml
DH$_2$O  99ml

7.4.4 Oil Red O
Oil Red O Stock
Oil Red O  0.5g
Isopropyl alcohol  100ml
Stand overnight

Dextrin
Dextrin  1g
DH$_2$O  100ml

Oil Red O working solution
Oil red o stock  60ml
Dextrin sol  40ml
Stand for 24hours +

7.4.5 Lipid Fixation

Lipid solution
Ethylene glycol  500ml
Linoleic acid  5g
Lecithin  2g
Stir for 1hour, let sit for 2+ hours
and draw off lower phase with
separatory funnel

2% Chromium trioxide
chromium trioxide  2g
DH$_2$O  100ml

Sodium bicarbonate solution
5% sodium bicarbonate  5g
DH$_2$O  100ml
7.5 Lipid fixation protocol validation

A large part of this project was investigating the suitability of incorporating a lipid fixation step, described by Tracy et al. to allow for paraffin embedded section to be taken and stained with oil red o.

Historically ORO staining is applied to frozen tissue processed using a cryostat. This technique is associated with increased artefact deposition which may potentially impact on diagnosis, but was necessary due to fat dissolution by the alcohols used in paraffin processing of fixed tissue. A technique championed by Tracy et al. in 2002 gives an alternative to frozen tissue processing by describing a paraffin sectioning technique which ensures lipid fixation in formalin fixed tissue. The technique uses a process which enriches the tissue with additional lipids specifically binding to the naturally deposited lipids within the sample. Exposure to chromic acid then fixes the lipids and allows for the introduction of fat soluble stains. This provides advantages and opportunities not afforded by frozen sectioning, such as the ability to cut sequential sections of tissue for specialty staining, allowing a myriad of tissue characteristics to be viewed in the same region of cells.

Liver tissue and tissues known to include lipids (skin + subcutaneous fat, visceral fat, kidney + visceral fat) were collected. All tissue samples were bisected with half fixed in formalin to be given lipid fixation treatment and the other half frozen for cryosectioning. Each would be stained using the same oil red o and H&E staining procedures and the quality of the stains compared in order to validate the lipid fixation protocol (Table 15).

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<td>5% sodium bicarbonate</td>
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7.5.1 Results

7.5.1.1 Lipid fixation attempt #1

Tissues were embedded in paraffin immediately after completion of lipid fixation. Embedded tissues were left overnight. The next morning all tissues had dried out resulting in shrunken and distorted tissue (Figure 34a). After re-embedding, tissue was cut and stained. Many of the sections cut had trouble remaining on the slide prior to and throughout the staining process (Figure 34b). Liver was the only tissue to remain on the slides but tissue damage was prevalent and staining appeared uneven and ill-defined, and cells appeared crowded (Figure 34).

7.5.1.2 Lipid fixation attempt #2

Immediately following lipid fixation tissues were processed overnight according to Table 2 (Page 27), embedded and sections cut. Architecture of the tissue was intact and staining was even, however lipids had been removed from the tissue during processing (Figure 35).

7.5.1.3 Lipid fixation attempt #3

In place of processing, the tissues were left to air-dry after lipid fixation. Tissues were placed between two biopsy pads within cassettes to prevent distortion (Figure 36). Once dry (after 4 days) tissue was placed within a wax bath for 3 hours at 60°C, embedded and cut.

Tissue, especially fat, was under processed and dissolved when placed on waterbath. Kidney and liver tissue successfully collected on slides was very damaged with uneven staining (Figure 37).

7.5.2 Conclusions drawn from failed attempts

Attempts at validating the lipid fixation protocol were halted after multiple attempts were made using the 3 alterations to the protocol outlined above. It appears processing is necessary after completion of the lipid fixation protocol as tissue consistency is not suitable for sectioning due to inadequate dehydration and paraffin penetration. However, post lipid fixation, the tissues lost all traces of lipid when processed due there dissolution by the alcohols and xylols applied.
Figure 34: Liver tissue post lipid fixation (attempt #1)

(A)

(B)

(C)

Figure 33: Liver dissection and sampling (A) Distorted liver tissue, post lipid fixation and embedding. (B) Sections of liver cut from the same tissue after re-embedding. Tissue can be seen lifting away from slide due to lack of processing. (C) The same liver sections stained with oil red o and photographed at x100 magnification. Tissue is cracked and cells are crowded.
Figure 35: Subcutaneous fat stained with oil red o (attempt #2)

(A) Subcutaneous fat processed following attempted lipid fixation (attempt #2). No lipids are visible as they have been removed during processing. (B) Subcutaneous fat which has been cryo-sectioned without processing. Lipids are visible in red. Both sections are stained with oil red o, photographed at x40 magnification.
Figure 36: Tissues left to air dry after lipid fixation (attempt #3)

Figure 37: H&E stained liver post lipid fixation with air drying, x40 magnification (attempt #3)
## 7.6 Suppliers list

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