The immune mechanism of developmental programming in non-alcoholic fatty liver disease

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This thesis is submitted to the University College London

For the Degree of Doctor of Philosophy

September 2018

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I, Jiawei Li, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

For my late grandfather, a wonderful maths teacher who first taught me the concept of number line.

Abstract

Previously, it was shown that maternal obesity before pregnancy, during pregnancy and lactation predisposes adult offspring for obesity and NAFLD, with the possible mechanism involving the breast milk. It was later shown that there is an interaction between maternal obesity and post-weaning diet, with alteration in Kupffer (liver macrophage) cells and natural killer cells in the liver.

As a sequel to previous findings in our groups, this project focuses on the programming effect of maternal obesity at earlier time points of development. We found that embryos from obese mothers had significantly lower body mass comparing to embryos from the lean mothers, accompanied by an increase in transforming growth factor-β (*Tgf-β*) and Glioma-associated (*Gli*) gene, and altered immune profile. Gli3 is a Hedgehog (Hh) responsive transcription factor, suggesting an involvement of the Hedgehog (Hh) Signalling Pathway in the developmental programming effect of NAFLD. Further investigation and published literature show that Hh is a crucial factor in modulating organ development and immune development. An adult mouse model in this project investigates the relationship between Hh and NAFLD. Taking all the factors together, this thesis demonstrates that there is an interactive effect between the Hh and obesity, which may be the underlying mechanism for the developmental programming effect seen in NAFLD.

Acknowledgement

Thank you to all those who have made this thesis possible. Firstly, I would like to express my immense gratitude to my supervisors, Dr Jude Oben and Prof. Tessa Crompton, for giving me this opportunity to work with them. From Jude, not only have I learned about hepatology, but I have also learned things beyond academic research, such as management, public health policy and charity work. Tessa's passion towards basic science research was infectious. Her creative ideas and attention to details are inspirations for my future career. Thanks to the UCL Grand Challenges for funding my project, and for the campaign of cross-disciplinary research.

Additionally, I would like to thank to Dr Paul Cordero, who has taught me not only how to work in a laboratory environment, but also the virtues of kindness and patience. I would also like to thank all members of the team: Anisha, Diana, Eleftheria, Rain, Sue and Vi. Also, thanks to the staff at the ICH biological services: Debbie, Ailsa, Kyle, Katie and Amy. Thank you very much for all your help, and patience in answering all my questions.

Thanks to my mother, a strong woman who always had faith in me, and sometimes believed in me more than myself. And my father who always reminds me the importance of patience, persistence and humility. And Jialu, for always encouraging me to carry on when the time got a bit tough and doing housework every day for the past months, so I can be in the right mindset

writing this thesis. Also, thanks to my three wonderful friends Aileen, Eliza and Rasini, who I have met from UCL Leading Women Society. Thank you for always listening to me tentatively, inspiring me constantly and dreaming with me daringly. All those hours we spent discussing our aspirations in London Grind café, will always be remembered and cherished.

Of course, I shall always remember that I would not be where I am without these crucial people in my life. Thanks to Dr Brenda Cross for allowing me the opportunity to take time off from my medical degree to pursue this PhD and opened my eyes to different possibilities. Thanks to Dr Anthony Antoniou and Prof Christopher Yeo, my previous supervisors from my summer research and my Bsc project respectively, who have been vital figures in getting me interested in scientific research.

Last and not the least, thanks to Nuffield Foundation for offering me the Summer bursary scheme back when I was 17, and thus initiated my interest towards science.

Impact Statement

Obesity is now the leading cause of health problems and diseases. Its pandemic level contributes to the rising prevalence of non-alcoholic fatty liver disease, such that it is likely to become the leading liver condition which requires liver transplantation, and very likely to overtake infectious liver diseases such as hepatitis B and C. This thesis contributes towards further understanding of the topic. All the knowledge, analyses, discovery or insight presented could be put to a beneficial use both inside and outside academia. This thesis is a continuation of the research work done by our group, to further the knowledge of the developmental programming mechanism of NAFLD. This could form a new subject area to be delivered in teaching. At the same time, this thesis would lead to academic publications and future research directions. Outside the academic world, the benefits this work provides are also insurmountable. The results could potentially lead to further collaborations with other sectors beyond academic community, including clinical use, public health awareness towards obesity and NAFLD, public policy design to encourage health eating and exercise, public service delivery to better allocate resources to population in needs, laws and quality of life. As obesity is a huge health problem both on a national and a global level, the impact from the research would be local, regional, nationally and internationally, to individuals, communities and organisations. Impact could be brought about through disseminating outputs in the form of academic publications, educational workshops for the general public, and engagement with public policy makers and public service delivery practitioners.

This thesis has resulted in two conference abstracts, which are being prepared

for academic papers:

- Li J, Cordero P, et al, Oben JA. Maternal Obesity Programs Offspring's Liver Immune Cells Intra-uterine. Journal of Hepatology. (2018); 68: S353-S254

- Li, J., Solanki, A., Cordero, P., Nguyen, V., Katar, C., Vinciguerra, M., . . . Oben, J. A. (2016). Interaction between maternal obesity and Hedgehog signalling influences immune cell development in the foetal liver. Presented at: 67th Annual Meeting of the American-Association-for-the-Study-of-Liver-Diseases (AASLD). **Received a prize of distinction.**

Contents

Abbreviations

Bp	Base Pair
ASMA	Actin Skeletal Muscle Alpha 1
ALT	Alanine Transaminase
AFLD	Alcoholic Fatty Liver Disease
ANOVA	Analysis Of Variance
AST	Aspartate Transaminase
BMI	Body Mass Index
BSA	Bovine Serum Albumin
CD	Clusters Of Differentiation
CMP	Common Myeloid Progenitor
cDNA	Complementary Dna
Dhh	Desert Hedgehog
DP	Developmental Programming
DN	Double Negative
E	Embryonic Day
FW:PW	Fetal Weight To Placental Weight Ratio
FITC	Fluorescein Isothiocyanate
FACS	Fluorescence Activated Cell Sorter
GGT	Gamma Glutamyl Transaferase
GWAS	Genome-Wide Assiciation Studies
Gli	Glioma-Associated Oncogene

GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GMP	Granulocyte-Monocyte Progenitors
Hh	Hedgehog
HSCs	Hematopoietic Stem Cells
HSC	Hepatic Stellate Cells
HFD	High Fat Diet
Ihh	Indian Hedgehog
iNKT	invarian Natural Killer T
KO	Knockout
LPS	Lipopolysaccharides
mRNA	Messenger Ribonucleic Acid
Mg	Microgram
Ml	Microliter
NK	Natural Killer
NKT	Natural Killer T
NAFLD	Non-Alcolholic Fatty Liver Disease
NASH	Non-Alcolholic Steatohepatitis
OPN	Osteopontin
Ptch	Patched
PCR	Polymerase Chain Reaction
Smo	Smoothened
Shh	Sonic Hedgehog
SD	Standard Deviation
SEM	Standard Error Of Mean

TNF-α   Tumour Necrosis Factor Alpha

WT   Wild Type

Chapter 1. General Introduction

1.1. Non-alcoholic fatty liver disease

Non-alcoholic fatty liver disease (NAFLD) is a serious liver condition which is often associated with obesity. It was first reported by Ludwig et al (Ludwig et al., 1980) and is often depicted as the hepatic manifestation of the metabolic syndrome. NAFLD ranges from hepatosteatosis (fat accumulation exceeding 5% of liver mass) to steatosis accompanied by inflammation known as non-alcoholic steatohepatitis (NASH) to cirrhosis. In some cases, it can lead to hepatocellular carcinoma (HCC) (Ganz et al., 2015, Younossi et al., 2015). HCC has become one of the most common cancer types and the third cancer related mortality worldwide, and the risk of liver cancer is increased by 17% and 89% in overweight and obese populations respectively (Larsson and Wolk, 2007). Histologically, NAFLD shares similar characteristics to alcoholic fatty liver disease, but only occurs in people who drink little or no alcohol (<30g for men, 20g for women per week) (2016). The risk factors include obesity, type II diabetes mellitus, hypertension and dyslipidaemia. The prevalence of NAFLD currently stands at 30% in the general population and 60.70% in diabetic patients (Sobhonslidsuk et al., 2015, Levene and Goldin, 2012). It is predicted to increase even further with the rising rate of obesity. Presently, NASH ranks the third among indications for liver transplantation in the United States (Perumpail et al., 2015). The increasing trend of NAFLD is also particularly worrying among the paediatric population, with NAFLD prevalence increasing in parallel to childhood obesity (Temple et al., 2016). The current childhood obesity prevalence is 12.6% globally, ranging from 5.4% (Finland) to 23.8% (China), and is predicted to increase even further (Broyles et al., 2015). It is

known already, that NAFLD occurs in 3% of children, reaching a staggering 80% in obese children (Singer et al., 2014). Another population that needs urgent attention is women of childbearing age. The obesity prevalence among this group is currently around 30%, and one in four women is obese at the time of pregnancy (Heslehurst et al., 2010). Besides causing gestational diabetes in the mothers, pregnancy loss and pregnancy complications, recent studies have shown that maternal obesity tends to predispose children to various metabolic diseases and cognitive dysfunctions, indicating that obesity is not just affecting the parents, but also their progeny. Therefore, urgent solutions, including research into the subject area, raising awareness and educating people at risk, are needed to combat this problem.

1.2. Obesity

The major cause of NAFLD is obesity, and the most internationally accepted criterion for quantifying obesity is the Body Mass Index (BMI), which is calculated as the weight in kilograms divided by the height in meters squared. A BMI of over 25 is defined as overweight and a BMI of over 30 is defined as obesity (Keys et al., 1972). Bioimpedence devices are sometimes used to estimate the percentage of body fat percentage. However, this technique is not a recommended, as the devices used to measure bioimpedence are often subject to great variations. Instead, the devices may be used to track the change of body fat percentage over time. Recently, many papers have proposed that BMI alone may not be indicative of obesity, that body fat

percentage and waistline circumference may provide additional information regarding the metabolic status, with waist circumference ≥94cm in male and ≥80cm in female considered as high risk (Janssen et al., 2004, NICE, 2014) **(Table 1).** Indeed, central adiposity is strongly linked to metabolically related conditions, such as type II diabetes, cardiovascular-disease and NAFLD (Alberti et al).

BMI classification	Waist circumference		
	Low	**High**	**Very high**
Overweight	No increased risk	Increased risk	High risk
Obesity 1	Increased risk	High risk	Very high risk
For men, waist circumference of less than 94 cm is low, 94–102 cm is high, and more than 102 cm is very high. For women, waist circumference of less than 80 cm is low, 80–88 cm is high, and more than 88 cm is very high			

Table 1. waist circumference provides extra clinical information in addition to the BMI values.

Presently, 25% of the UK population is classified as obese and this was predicted to reach 51% by 2030 if the current trend of increase continues.

Furthermore, obesity was predicted to cost $549.50 billion on medical expenditure cumulatively between 2010 and 2030. If indirect costs resulting from obesity are included, the total cost would be even more (Finkelstein et al., 2012). Obesity is accompanied by a chronic state of inflammation with many associated comorbidities such as insulin resistance, hypertension, hypertriglyceridemia or low HDL cholesterol among others (Gonzalez-Muniesa et al., 2017). The combination of these conditions is known as metabolic syndrome. In fact, NAFLD has been suggested to be the liver manifestation of metabolic syndrome (Cordero et al., 2017). In some cases of NAFLD, the patients are not obese according to the definition of BMI; however, 80% of obese patients have NAFLD; and conversely, out of the NAFLD patient population, 90% are known to be obese (Williams et al., 2011). Therefore, there is a close and undeniable relationship between NAFLD and obesity. The rapid rise in obesity prevalence is largely due to lifestyle factors, which include the increased consumption of high energy food, and also the sedentary lifestyle of modern society. However, the dramatic change in prevalence cannot be explained by lifestyle factors alone. Other elements may also play a major role in the phenomenon.

1.3. Diagnosis

There are currently no definitive diagnostic tools for NAFLD, and the diagnosis is largely by exclusions. Serological parameters, such as aspartate transaminase (AST), alanine transaminase (ALT) and gamma glutamyl

transferase (GGT) have been used in NAFLD diagnosis; however, these tools often lack sensitivity and specificity. AST to ALT ratio is used as an indicator of NAFLD, with AST/ALT less than 1 in NAFLD, but greater than 1 in AFLD (Chalasani et al., 2012). However, in some cases, ALT levels are found to be normal despite the presence of hepatosteatosis. Ultrasonography is also used in the diagnosis of NAFLD, which categorises the extent of the condition according to severity: mild, moderate and severe, but similarly, this technique lacks sensitivity and specificity. An alternative to conventional ultrasonography is Transient elastography (Fibroscan), which detects the stiffness of the livers in patients (Cassinotto et al., 2016). So far, the most reliable diagnostic tool is biopsy; however, this method comes with the problems of pain and other complications due to its invasive nature (Spengler and Loomba, 2015). Histologically, NAFLD has the hallmarks of macrovesicular steatosis, lobular inflammation, hepatocellular ballooning, lytic necrosis, apoptotic bodies, portal inflammation and microgranuloma. Upon staining with Haematotoxin & Eosin, NAFLD often displays inflammatory cell infiltrates and Mallory's hyaline (Yeh and Brunt, 2014). Therefore, biopsy of patients, followed by histological evaluation by a pathologist remains the gold standard. Normally, the pathologist would score the biopsies, using the NAS score system and fibrosis staging.

NAS Score

Item	Score	Extent
Steatosis	0	<5%
	1	5-33%
	2	>33-66%
	3	>60%
Lobular Inflammation	0	No foci
	1	<2 foci/200X
	2	2-4 foci/200x
	3	>4 foci/200x
Hepatocyte Ballooning	0	None
	1	Few balloon cells
	2	Prominent ballooning
Chronic portal inflammation	0	None
	1	Mild
	2	>Mild

Fibrosis staging

0	None
1	Perisinusoidal or periportal
1A	Mild, zone 3, perisinusoidal
1B	Moderate, zone 3, perisinusoidal
1C	Portal/periportal
2	Perisinusoidal and portal/periportal
3	Bridging fibrosis
4	Cirrhosis

Table 2. NAS greater than 5 is classified as NASH, 4 indicates cirrhosis (Brunt et al., 1999, Brunt, 2005).

The search for a more reliable, non-invasive marker for NAFLD is still ongoing. Elevated fibrogenesis markers have been suggested, given that both animal

models and humans have elevated actin skeletal muscle alpha 1 (ASMA), transforming growth factor beta (TGF-beta) and collagen type I and II (COL1A1 and COL2A1) from blood serum or liver sections.

1.4. Management

Besides treating the liver condition specifically, it is important to also manage the associated co-morbidities: obesity, hyperlipidaemia, and Type II diabetes. So, controlled weight loss with diet change and physical exercise remain the most effective therapeutic methods for NAFLD (Chalasani et al., 2018). There are certain drugs targeting NAFLD, however, the pharmaceutical approach should only be considered when the patients have biopsy proven NASH and fibrosis. For these drugs, the mains roles are often to protect liver damage, and include, insulin sensitizer, antioxidants and anti-TNF-α. Anti-obesity pharmacotherapies, such as Orlistat, Sibutramine and Rimonabant, are also used in some cases (Zelber-Sagi et al., 2006, Sabuncu et al., 2003, Wierzbicki et al., 2011), with the purpose of inhibition of fat absorption, inhibition of the endocannabinoid system, and/or modification of the central nervous system.

Bariatric surgery, currently only used as a therapeutic measure for obesity, has been implemented as a viable method of management for NAFLD, and evidence from a cohort of NASH patients has shown reduced liver inflammation level and improved liver transaminases resulting from this

surgical procedure (Bower et al., 2015). The primary aim of bariatric surgery is to reduce the volume of stomach, and increase satiety, thus decreasing either the food intake or the calorie retention. Nowadays, the majority of the bariatric operations are performed laparoscopically, and therefore, are minimally invasive. However, the use of bariatric surgery as a treatment of NAFLD is still under evaluation. Furthermore, non-surgical interventions such as intragastric balloon has been recently studied with promising results as a novel treatment for NAFLD (Nguyen et al., 2017).

1.5. Pathogenesis

Besides obesity, genetic background also appears to have a key role in the pathogenesis of NAFLD. Firstly, there appears to be an ethnic component, indicative of genetic influence. Hispanic populations are at higher risk of developing NAFLD, and African descendants appear to be protected from the condition, regardless of diabetes or obesity. Familial studies also suggest a familial clustering of NAFLD and NASH (Caussy et al., 2017) and twin studies also suggest a genetic component in NAFLD, as hepatic steatosis correlated between monozygotic twins but not between dizygotic twins (Loomba et al., 2015). In addition, recent genome-wide association studies (GWAS) identified major genes related to hepatic fat accumulation: palatin-like phospholipase domain-containing 3 (PNPLA3) gene and transmembrane 6 superfamily

member 2 (TM6SF2) gene. These two genes are both related to lipid droplets remodelling and VLDH secretion (Del Campo et al., 2018).

There is also a close relationship between NAFLD and diabetes. Many studies were undertaken to investigate the natural history and the relationship between the two conditions. It has been reported that 60.70% of diabetic patients have fatty liver disease (Sobhonslidsuk et al., 2015). It is not clear which occurs first in patients, and some studies have proposed that type II diabetes is a prerequisite of NAFLD (Williams et al., 2013).

One leading hypothesis of NAFLD pathogenesis is the '*two-hit hypothesis*'. The first hit involves accumulation of fat in the liver due to excess free fatty acids or enhanced *de novo lipogenesis*, thus rendering the liver vulnerable to further damage. The second hit involves oxidative stress, cytokine-mediated inflammation and insulin resistance (Day and James, 1998). This theory later developed into a *multi-hit hypothesis* by Tilg and Moschen, suggesting that the manifestation of this condition is a multi-factorial process, with these candidate processes running in parallel: (1) Intrinsic liver lipotoxicity; (2) change and dysbiosis of gut microbiota; (3) gut derived endotoxin (e.g., lipopolysaccharides) due to increased gut permeability; (4) activation of the innate immune system due to the interaction of toll-like receptor ligands with endotoxins; (5) inflammation of extrahepatic organs such as adipose tissue;

(6) unfolded and/or misfolded proteins, leading to cellular stress pathways; and (7) adiponectin secretion (Tilg and Moschen, 2010).

1.6. Developmental programming in NAFLD

In line with the *multi-hit hypothesis*, the first 'hits' start during early stages of development. Research is underway to test the theory that the pathogenesis of NAFLD may be explained by the phenomenon known as the developmental programming (DP). This theory, also known as the theory of the Developmental Origin of Health and Disease (DOHaD) was first proposed by David Barker, a British epidemiologist who observed a strong correlation between low birth weight and development of cardiovascular diseases in adulthood (Barker and Osmond, 1986b). This theory, also known as 'Barker's hypothesis', proposes that exposure to environmental stimuli during a plastic period of life may impact the risk of certain chronic health conditions during later life. This hypothesis was further supported by Roseboom et al, who drew a link between maternal under-nutrition during gestation and disturbed metabolic profiles in offspring (Roseboom et al., 2006). The same principle from observations of conditions of under-nutrition was also implicated in studies on over-nutrition, and it was also observed that maternal over-nutrition predisposes to certain conditions; maternal exposure to a high-fat, high protein diet led to obesity, impaired glucose metabolism and insulin resistance in offspring (Campbell et al., 1996, Villamor and Cnattingius, 2006). This phenomenon may be explained by the thrifty hypothesis, that the

developmental programming is a consequence of the nutritional insults, which was supposed to maximise survival advantage (Hales and Barker, 2001). However, this phenomenon evoked a predictive adaptive response, which led to a mis-match between metabolic load and postnatal offspring metabolic activity, due to the changes in nutritional patterns in modern society (Bateson et al., 2014). The essence of developmental programming is that the effects take place in one generation, indicating that the mechanism consists of epigenetic mechanism, rather than genetic mutation. The regions often affected are sections of DNA abundant in CpG islands, which can be unmethylated, methylated or differentially methylated to change the expression of the gene (Jones, 2012) **(Figure 1)**.

(Adapted from Qiu J 2006 Nature, Cordero P 2010 Rev Esp Obes)
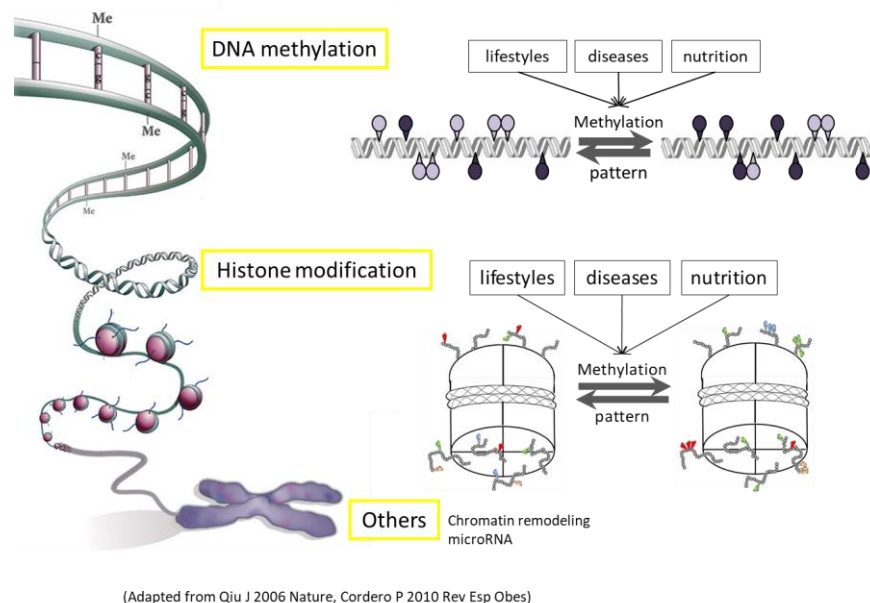
Figure 1: Epigenetic mechanisms involved in developmental programming.

For NAFLD, the evidence is strong that maternal obesity predisposes offspring to this condition, with the consensus that there are three major windows of opportunities during human development, where insults and stimuli could lead to of the effect of developmental programming in NAFLD. These are conception, perinatal period and postnatal period **(Figure 2)**. As NAFLD is known as a health condition which often manifests later in adulthood, many of the DP studies are either retrospective studies from historical clinical records or from animal models with rodents and non-human primates as the chosen candidates. The Western Australian Pregnancy (Raine) cohort study of 1170 adolescents showed that maternal obesity and higher gestational gain are associated with NAFLD in female offspring (Ayonrinde et al., 2018). Many animal studies attempt to mimic the human condition by inducing obesity in mothers before conception, and then observe the characteristics of the adult offspring. Guo and Jen (1995) first reported in rodents that maternal over-nutrition led to hepatomegaly.
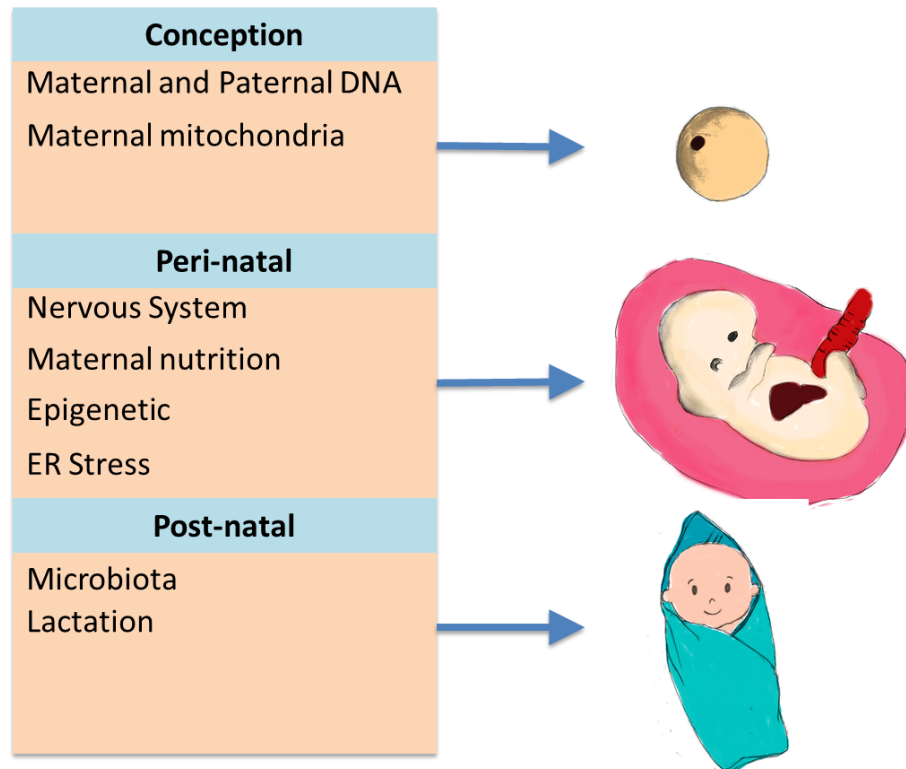
Figure 2 Critical windows for the effect of developmental programming.

During development, insults and stimuli could take place during any or all these periods.

1.6.3. Mitochondria involvement

The involvement of mitochondria in the developmental programming of NAFLD has been suggested, as they are involved in oxidative stress and energy utilisation. This is particularly interesting, as the mitochondria are not only responsible for metabolism and lipid process, but are also inherited maternally alone, which poses the question of whether maternal dietary status may influence offspring by affecting mitochondria (Sato and Sato, 2012). Indeed, it

has been reported that exposure of offspring to maternal high fat diet (HFD) induced mitochondrial dysfunction without significant changes in adiposity of the animals, accompanied by elevated mitochondrial Complex I and II activation, reduced mitochondrial cytochrome c and increased glutamate dehydrogenase (Alfaradhi et al., 2014). 'Blobby mice', which are transgenic mice with mutation at *Alm1* gene, were shown to exhibit an obese phenotype, despite being on normal chow (Wu et al., 2015). The oocytes of these mice have reduced mitochondrial membrane potential and high levels of autophagy, yet normal levels of mitochondrial membrane potential. This suggests that alterations take place even before fertilisation. Furthermore, the blastocytes from 'blobby mice' had reduced mitochondrial DNA content (Wu et al., 2015).

1.6.4. Importance of lactation in developmental programming

Many studies have highlighted the importance of lactation on the influence of obesity and NAFLD in offspring. Oben et al (2010a) then utilised a novel mouse model of maternal programming, involving cross-fostering immediately after birth between lean mothers and obese mothers, and confirmed that maternal obesity programs the development of NAFLD, with the lactation period being the crucial window. Bayol et al (2010) reported that obesogenic diet during pregnancy and lactation promoted NAFLD in offspring. We have previously shown that breast milk from obese mice has increased concentration of leptin, which is correlated with obesity in offspring. Similar observations were also found in human studies showing that there is a positive correlation between

leptin concentration in breast milk consumed by the infant and the infant weight gain between 1 month and 3 months old, with no significant correlation between maternal BMI and amount of leptin in the breast milk (Kon et al., 2014).

1.6.5. Involvement of the innate immune system in developmental programming

Mouralidarane et al later developed a model, where offspring from obese mothers were weaned onto either control or obesogenic diet and showed that offspring which were born from obese mothers and weaned onto the same diet had much higher risk and greater severity of NAFLD associated with innate immune dysfunction, such that maternal obesity leads to increased Kupffer cells (KC) number but decreased KC phagocytic function. In the same study, the number of natural killer T (NKT) cells was decreased. NKT cells appear to be positively correlated with reduced fibrosis, thus exerting a protective role (Mouralidarane et al., 2013). It is thought that NKT cells inhibit liver fibrosis by phagocytosing activated hepatic stellate cells (HSC) directly (Mitra et al., 2014). Later in non-human primates, it was shown that maternal high-fat diet triggered lipotoxicity in the foetal livers (McCurdy et al., 2009). Similarly, Bouanane et al reported that maternal obesity permanently reduced hepatic ß-oxidation and increased lipogenesis (2010).

1.7. The immune system and its role in NAFLD

The text book definition of immunity in vertebrates consists of a spectrum of responses, with the innate response at one end which is more crude and fast, and the adaptive response at the other, which is more specific and adapts across the life-time of the individual (Ziauddin and Schneider, 2012). Rodents, particularly mice, are used to study the development of immune system, as the mouse model shares more similarities to humans, and can be modified and manipulated genetically, providing an effective model for the human immune development (Mestas and Hughes, 2004).

The liver has a close relationship with the immune system, such that the liver is often considered as a major immune organ, and sometimes even dubbed the 'fire-wall' of the human body (Balmer et al., 2014), due to the fact it contains a large number of immune cells: Kupffer cells (KC) which are resident macrophages of the liver accounting for 80% of immune cell number. HSC can also act as antigen presenting cells exhibiting innate immune functions. There are also many dendritic cells and other non-lymphoid cells in the liver. All these cells are a part of the classic innate immune system, which contributes to hepatic inflammation, fibrosis and cirrhosis. HSC are also a major storage site for vitamin A, which contributes to immunity regulation in the liver (Li et al., 2016). There is strong evidence that HSC produce inflammatory cytokines,

and reactive oxygen species, thus interacting with other immune cells (Puche et al., 2013).

The immune cell type mostly linked to NAFLD are macrophages (in this instance known as Kupffer cells); NK cells; NKT cells; and neutrophils. Kupffer cells, also named Ito cells, are the resident macrophages in the liver. They circulate in the sinusoid space, and are mainly responsible for clearing endotoxins, debris and microorganisms (Wenfeng et al., 2014). They also have the role of modulating the differentiation and activation of other immune cells. KCs are activated by pathogens or damage associated molecular patterns (PAMPs and DAMPs). KCs respond to IL6 (proinflammatory) and IL10 (anti-inflammatory), with the balance depending on the duration of *Signal transducer and activator of transcription 3 (STAT3)* gene activation: transient activation favours a pro-inflammatory response and prolonged activation favours an anti-inflammatory response. For the pathogenesis of NAFLD, IL6 and IL10 also play an important part in the onset and progression of the condition (Sharma et al., 2015). Furthermore, ablation of KC improved histological outcome of NASH (Rivera et al., 2007). Studies have shown that KC functions are impaired in NAFLD models without a decrease in KC number. It was also shown in a rat model, in which rats were subjected to a methionine and choline deficient (MCD) diet (a widely used diet-induced NASH model) that the number of KC increased but there was an impairment of their function with altered KC morphology (Asanuma et al., 2010). As the liver Kupffer cell population is

established prior to birth and self-maintained post-natally, it would be interesting to see whether it is affected by maternal obesity.

Liver-specific NK cells were first described by Wisse et al in 1976. They exhibit different characteristics from peripheral NK cells, immunophenotypically, functionally and morphologically (Wisse et al., 1976). NKT cells are a unique subset of NK cells, which exhibit both surface receptors specific for natural killer cells and T cell receptors (TCR) (Tajiri and Shimizu, 2012). NKT cells are generally depleted in liver steatosis but increased in NASH related liver fibrosis (Syn et al., 2010). NKT deficient mice fed an MCD diet, showed blunted Hedgehog and osteopontin (OPN) expression and decreased fibrogenic factors (Syn et al., 2012). Kremer et al (2010) reported that in patients with NASH, biopsies showed a depletion in NKT cells numbers correlated with the severity of hepatosteatosis suggesting that NKT cells might play a role in attenuating the fibrosis level in the liver. Gao and Radaeva (2013) also suggested that the NKT cells may protect against liver injury, but only when the NKT cells are weakly activated. Yet under strong activation, NKT cells may promote liver inflammation and injury. Wang and Yin (2015) as shown that NK/NKT cells enhance tumour metastasis in the liver.

Neutrophils have been suggested to be involved in the early phase of adipose tissue inflammation. The oxidant-generating neutrophil enzyme, known as Myeloperoxidase (MPO) is involved in steatosis of the liver, where the MPO

was suggested to induce insulin resistance (Xu et al., 2014). Recently, several articles have proposed the use of neutrophils as a potential biomarker for NAFLD (Alkhouri et al., 2012, Ozturk et al., 2015, Abdel-Razik et al., 2016).

Other immune cells have also been implicated in NAFLD. Mast cells are often associated with an allergic reaction and usually found in white adipose tissue, yet deletion of mast cells has been shown to reduce body weight gain, inflammatory cytokines and chemokines (Jarido et al., 2017). These cells may play a role in the pathogenesis of liver fibrosis. Additionally, a proinflammatory state of dendritic cells (DC), the professional antigen presenting cells usually found around the central and portal veins is associated with liver fibrosis, whereas the depletion of DCs *in vivo* limits NASH-related fibroinflammatory injury (Almeda-Valdes et al., 2015).

There appears to be less involvement of the adaptive immune system in the pathogenesis of NAFLD, as the total population of hepatic lymphocytes is relatively stable in NASH. However, an increased ratio of CD8/CD4 cells has been described in the liver (Ma et al., 2016). As for the other group of adaptive immune cells, B cells, these contribute to around 6% of intrahepatic immune cells. NASH patients were shown to have an increase in the serum level of B-cell activating factor (BAFF), and BAFF receptor deficient mice show an improved profile when subjected to high fat diet (Zhang et al., 2016).

From an immunological perspective, the immune system can be represented as a continuous spectrum instead of being two distinctive cell populations, with innate cells at one end and adaptive cells at the other, with cross talk between the innate immune cells and adaptive immune cells. It has been shown that the deletion of CD8+ cells reduced macrophage infiltration, which in turn ameliorates the liver phenotype in NAFLD (Nishimura et al., 2009).
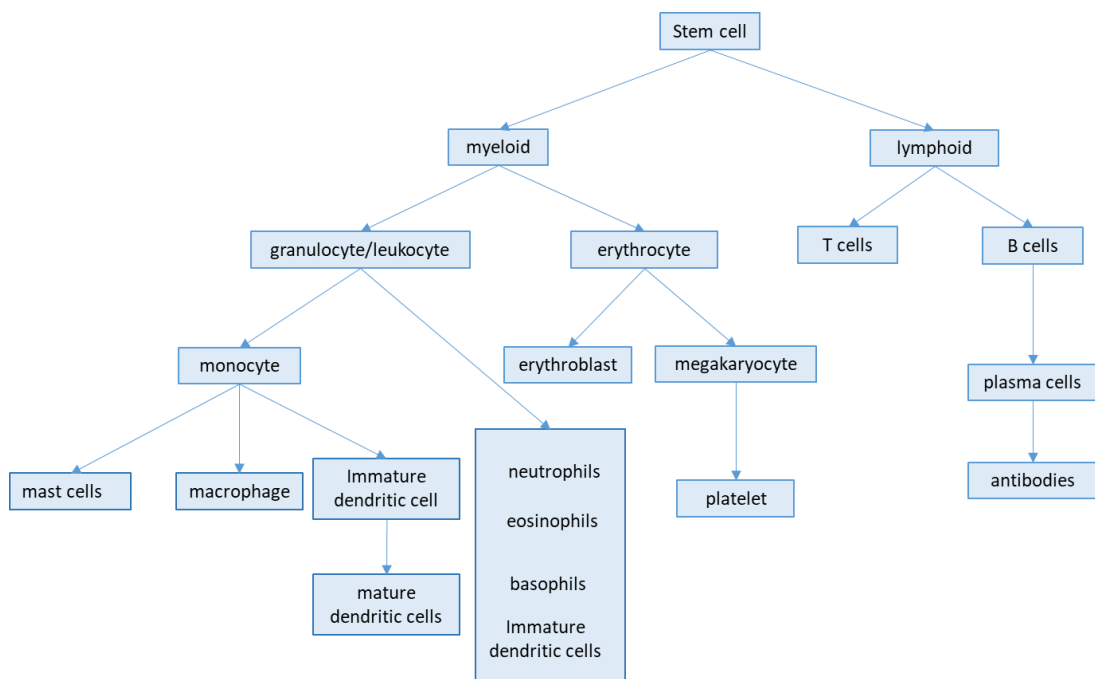
Figure 3: Immune cell development.

1.8. Developmental programming of NAFLD and the plastic window of the immune system

The development of the immune system is a complex process of blood formation (known as haematopoiesis) **(Figure 3)** and immune cell maturation, which involves the migration of immune cells to different anatomical structures in temporal sequences. There are two sequential waves: primitive and definitive. In mice, primitive haematopoiesis takes places in the yolk sac at Embryonic Day 7.5 (E7.5) beginning with production of primitive erythroid progenitors, generating a population of haemapoietic stem cells (HSCs). All immune cells originate from the common HSCs progenitors. Definitive haematopoiesis starts in the aorta gonad-mesonephros (AGM) region at E10-11, and then migrates to the foetal liver to continue throughout gestation (Jagannathan-Bogdan and Zon, 2013) **(Figure 4)**. The progenitor cells differentiate in a hierarchical order, leading to a cascade of more differentiated and restricted progenitors (Cumano and Godin, 2007). The early stages of the immune cells are mechanistically defined by their response to specific cytokine stimulation. As the cells mature, they can be classified phenotypically by their surface receptors (Golub and Cumano, 2013). The HSCs later give rise to common lymphoid progenitors and myeloid progenitors. The myeloid progenitor cells are the precursor of the granulocytes, macrophages, dendritic cells and mast cells of the innate immune system, which are also known as the more mature forms of monocytes (Terry and Miller, 2014). The lymphoid lineage gives rise to cells involved in the adaptive response, with B cells and T cells as the major lymphocyte types.
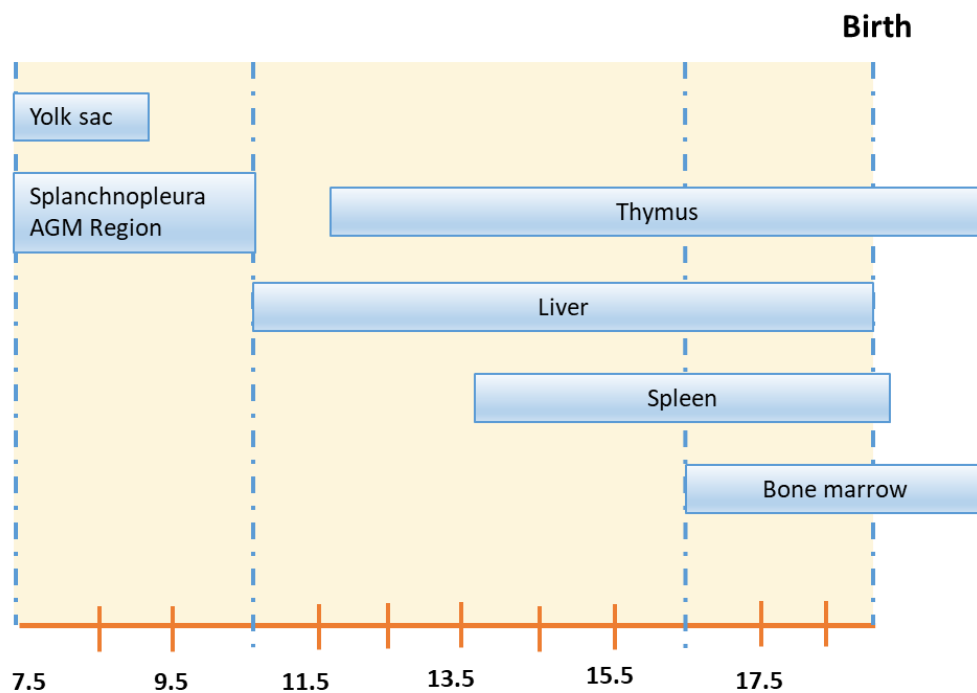
Figure 4.Haematopoiesis and embryonic development in mice.

Two common features linking the immune system and the developmental programming theory, are that both are highly plastic and modifiable at early stages of vertebrate development, due to the complicated immune developmental pathways, and the sequential, hierarchical manner of immune cell formation, which provide windows of opportunity for changes to take place **(Figure 5)** (Landreth, 2002) . Several factors can steer the direction of development during this period: developmentally important signalling pathways, such as the Hedgehog signalling (Hh) pathway and Wingless (Wnt) pathway; the diversity of microbiome and exposure to different cytokines.
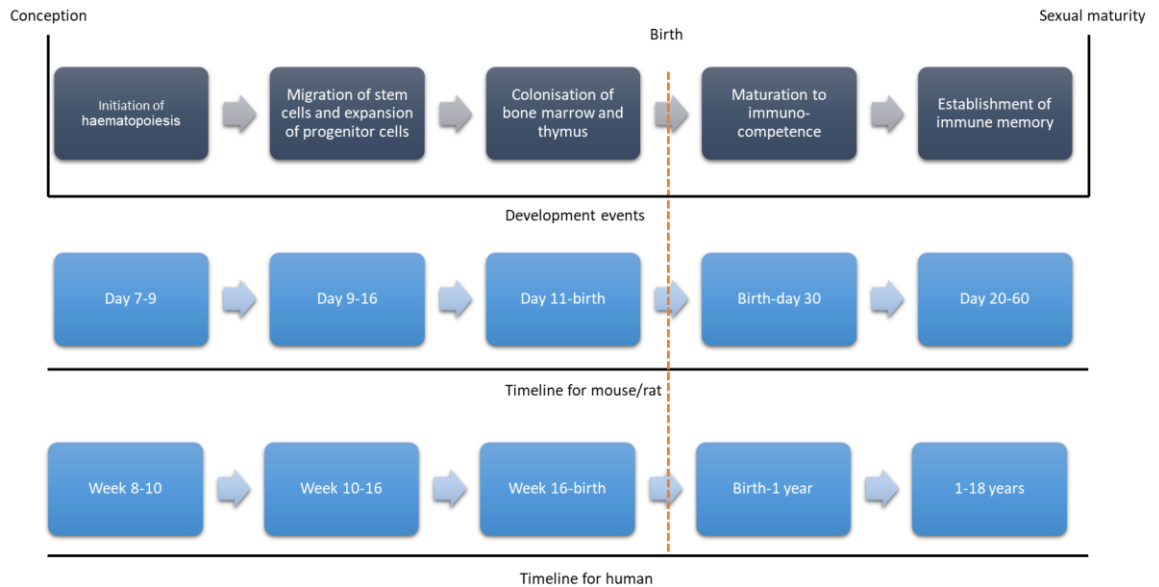
Figure 5. Five critical windows of immune development.

According to Dietert, there are five critical windows of potential vulnerability, with three before birth, and two after birth: (1) when haematopoiesis first started; (2) during migration of cells and expansion of progenitor cells; (3) colonisation of bone marrow and thymus; (4) maturation of immune-competence and; (5) establishment of immune memory (adaptive immune system). The figure also shows the parallel timeline of development of both rodents and human.

1.9. Hedgehog

The Hedgehog (Hh) signalling pathway was first identified in *Drosophila* by large-scale screen for mutations, but was later found in all vertebrates (Nusslein-Volhard and Wieschaus, 1980). It is an important signalling pathway

responsible for organogenesis in embryos and tissue regeneration in adults and exert effects onto target cells and tissue depending on the concentration of the proteins and the duration of exposure. There are three mammalian types of Hh proteins: Sonic Hedgehog (Shh), Indian Hedgehog (Ihh) and Desert Hedgehog (Dhh), with Shh being the most well studied ligand. All of them can work in short- and long-range (Ingham and McMahon, 2001).

Hh functions through two types of pathways: the canonical pathway and non-canonical pathway. The canonical pathway is a complex signalling cascade, dependent on primary cilia, which are small immotile cilia located on most cells (Briscoe and Therond, 2013) **(Figure 6)**. Hh proteins are released as a precursor with molecular weight of 45kDa, which then auto-cleaves to give rise to two signalling domains: Hh N-terminal (Hh-N) and Hh C-terminal (Hh-C), with molecular weights of 20kDa and 25kDa respectively. Hh-N is the active component, whereas the function of Hh-C is currently unknown. Hh-N binds to Patched-1 (Ptch1), a 12-span transmembrane protein which normally inhibits another 7-span transmembrane protein-Smoothened (Smo). The binding of Hh-N to Ptch1 disinhibits Smo; consequently, Smo is translocated to primary cilia leading to activation of the Glioma-association (Gli) family of transcription factors. There are three Gli proteins: Gli1, Gli2 and Gli3. Gli1 and Gli2 act as activators and Gli3 acts mostly as a repressor of gene transcription (Jacob and Lum, 2007). As Gli3 is mainly a repressor, the absence of this gene leads to a global increase in the Shh signalling. It has been shown that the Hh signal is correlated with the concentration of Gli proteins and that the balance between

Shh and Gli3 contributes to organogenesis and pattern formation during brain and limb development (Motoyama, 2006). Two types of non-canonical Hh signalling have been described: Type 1 is Ptch-dependent and Smo independent, and type 2 is smo-dependent, but Hh does not interact with Ptch.
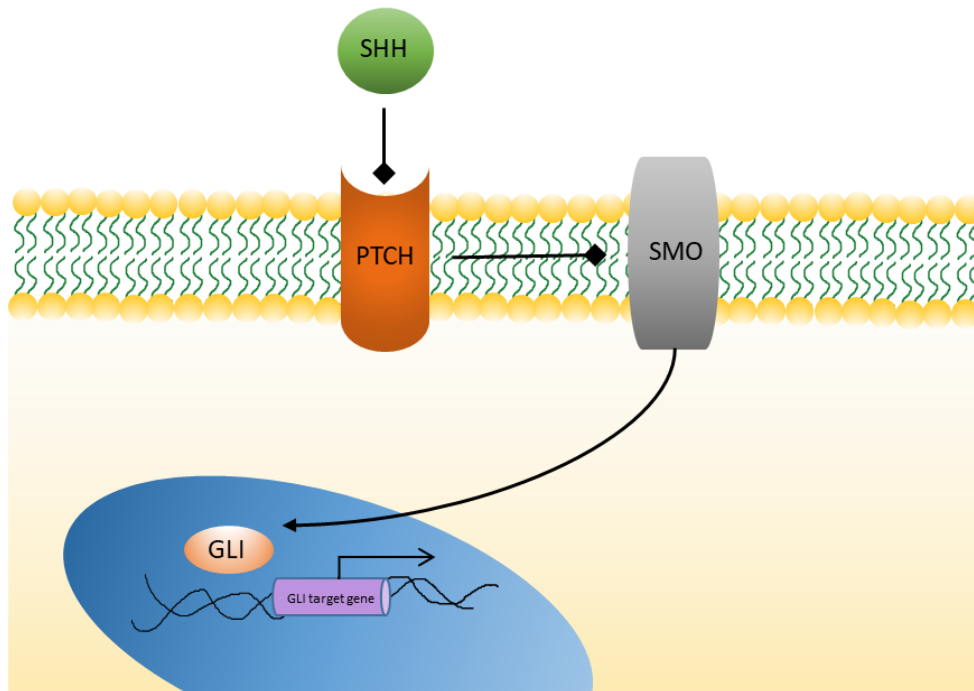
Figure 6. the canonical pathway of the Hedgehog signalling pathway.

Shh acts to inhibit Ptch, consequently removing the inhibition of Ptch on Smo. Smo is then allowed to translocate to the nucleus to activate Gli, so the Gli target genes are transcribed.

1.9.1. Hedgehog pathway and the immune development

Hh has an important role in the development of immune cells. Detmer et al (2005) has showed that Hh is involved in the control of haematopoiesis, where it was shown that co-culturing HSCs with Shh inhibitor cyclopamine negatively regulated erythroid maturation, whereas, co-culturing HSCs with Shh agonists increased the proliferation and differentiation of granulocytes and erythroids. A similar observation was made by Bhardwaj et al, that cell proliferation in primitive haematopoietic progenitors increased significantly when they were treated with soluble forms of either Shh or Dhh (Bhardwaj 2001). Outram et al showed that the signalling regulates T cell development in the thymus and Shah et al also showed that in Shh-/- embryos, there is reduced thymocyte cellularity and differentiation (Shah et al., 2004, Outram et al., 2000). In the foetal livers, Shh has recently been shown to regulate B cell development (Solanki et al., 2017). As the thymus is the primary site of T cell development in embryos, impairment of thymocyte development might have a long-lasting impact on immune cells in adulthood. Thymocytes are not the only cells affected by Hh pathway. Lau et al showed that Dhh plays a negative regulatory role in erythroid differentiation (Lau et al., 2012). There are fewer studies on the role of Hh in the innate immune system, with the majority of experiments in the context of myeloid disorder, for example acute myeloid leukaemia and chronic myeloid leukaemia (Khan et al., 2015). It was reported that deletion of PTCH1 in the bone marrow increased mobilisation of myeloid progenitors, and Gli1 deficient mice were shown to have decreased proliferation of Common

Myeloid progenitors (CMP) (Merchant et al., 2010) and Dhh-deficient mice had fewer GMP and more EMP (Lau et al, Blood 2012).

1.9.2. Hedgehog pathway and adipogenesis

The Hh pathway inhibits adipogenesis in a conserved manner. One study showed that Hh signalling inhibits fat formation in Drosophila (*in vivo*) and mouse (*in vitro*), and this is probably by acting upstream of peroxisome proliferator-activated receptor gamma (PPARγ), a key regulator of the adipocyte lineage (Suh et al., 2006). Additionally, in the same study, it was observed that mice with diet induced obesity had decreased expression of the Hh signalling pathway. Similarly, it has been shown in isolated human multipotent adipose-derived stem (hMADs) cells, that activation of the Hh pathway impairs adipogenesis, decreases the size of adipocytes and reduces insulin sensitivity (Fontaine et al., 2008). A recent report showed that mice with a germline mutation of BOC which is an important coreceptor in the Hh pathway during embryonic development, displayed increased adiposity, abnormal glucose homeostasis and decreased insulin sensitivity; furthermore, all the observations were exacerbated by feeding high-fat diet (Lee et al., 2015). Taken together, Hh downregulates adipogenesis and obesity and may also reduce hepatosteatosis in NAFLD.

1.9.3. Hedgehog pathway and the Liver

Hh pathway plays an important role in the liver and exerts autocrine and paracrine actions in adult and foetal livers (Ingham and McMahon, 2001). During liver organogenesis, Shh is expressed in the ventral foregut endoderm from which the liver derives. The expression disappears at onset of the liver bud formation (around E9.5) in a mouse model, suggesting that Hh pathway might need to be shut off, or drastically reduced, for normal differentiation of hepatoblasts. In mutant mice, the formation of liver bud still occurs in Shh-/- and Gli2-/-, Gli3-/- at E9.5, but the size of the liver is smaller (Ingham and McMahon, 2001), and Shh and Gli3 regulate B cell development in the foetal livers (Solanki et al., 2017).

One leading theory of the pathogenesis of NAFLD is the endotoxicity induced by microbiota. This theory hypothesises that the microbiota alter the gut permeability, allowing increased levels of endotoxic ligands such as lipopolysaccharides (LPS) to reach the liver, thus leading to NAFLD. One study draws a link between Shh and LPS, such that mouse with reduced receptors for Shh were shown to be more resistant to LPS induced endotoxicity.

1.10. Hedgehog Pathway: the missing link between immunity, developmental programming and NAFLD?

Many studies have suggested a relationship between Hh and NAFLD. When the liver is injured, Hh is reactivated for liver regeneration. One study indicated that Hh signalling may lead to the activation of adult quiescent hepatic stellate cells (qHSC) into activated HSC (aHSC) through the interaction with Rac-1 receptors on HSC, consequently leading to cirrhosis (Choi et al., 2010). Therefore, the link between Hh pathway and immune cell development may provide a clue for a possible link between Hh pathway and development of NAFLD. There are currently no published papers investigating the role of the Hh pathway in programming of NAFLD. As the Hh pathway has major roles in organogenesis, morphogenesis and immune regulation, it would be interesting to investigate whether maternal obesity, used in an established NAFLD programming model, interacts with the Hh pathway to impact development of offspring, thus affecting their liver status. Additionally, our previous studies have focused on studying adult offspring, yet no studies have been carried out on the inter-uterine stage. Therefore, this project aims to investigate the development of foetal livers using a mouse mutant of the Hh pathway, subjected to maternal obesity intra-uterine. Additionally, it is known that there are high number of Hh producing and responsive cells in early stages of development and the number decreases with age (Lee et al., 2016).

Previous studies from our group demonstrated a strong link between NAFLD and maternal programming of offspring, possibly involving an innate immune mechanism, and that the offspring subjected to maternal obesity during pregnancy and lactation exhibited a significant increase in the number of Kupffer cells (KC), but decreased KC phagocytic functions. Natural killer T (NKT) cells, another important type of lymphoid cells in the liver, were observed to show a decrease in number in the same study. There is strong evidence that Hh is involved in immune cell maturation and differentiation (Crompton et al., 2007). Shh and Ihh knock out embryos showed reduced thymocyte cellularity and differentiation (Shah et al., 2004) (Outram et al., 2009. In the foetal liver, Gli3 knock out embryos show decreased B cells development whereas Shh knock out embryos have increased B cell populations (Outram et al., 2009).

As the thymus is the primary site of immune progenitor cell development in embryos, impairment of thymocyte development might have a long-lasting impact on the immune cells in adulthood. Indeed, dendritic cells, T-cells and NKT cells are affected by the absence of Hh signalling pathway. Therefore, the link between Hh pathway and NKT cells provides a clue for a possible link between Hh pathway and the programming of NAFLD.

Although in programming models, the evidence is clear that stimuli during the pregnancy and lactation periods have major impacts on offspring health, the

analysis was limited to offspring post-natally, or during adulthood. There are currently no published studies investigating the role of Hh pathway in programming of NAFLD. As the Hh pathway has major roles in organogenesis, morphogenesis and immune mechanism, it would be interesting to observe whether peri-natal changes in the Hh pathway have an impact on foetal livers. The aim of this project is to investigate the development of mouse foetal livers at different time points during pregnancy with and without the presence of hedgehog signalling pathway, based on our existing animal model of maternal obesogenic diet.

General Hypotheses:

Our group has previously shown that maternal obesity predisposes offspring to NAFLD, through involvement of the innate immune system with alterations in KC and NKT profile. This thesis proposes that the programming effect might occur as early as the embryonic stage and that the Hedgehog signally pathway may be involved in shaping the immune system of offspring exposed to maternal obesity.

Maternal obesity affects the development of the immune system during the intra-uterine stage, thus predisposing offspring for obesity and NAFLD. This alteration may be caused by the factors between the maternal and foetal interphase. We have decided to investigate the involvement of Hedgehog

Signalling Pathway, because of its close association with organogenesis, and the evidence of its important role in adult and foetal livers.

This project sets out to be a continuation of our previous work undertaken but trace a much earlier time point of development. Based on the premises set out by the hypothesis that metabolic syndrome originates from foetuses; I will investigate the following hypotheses:

Hypothesis 1 (Chapter 3): Maternal obesity impacts on the offspring as early as the intra-uterine stage, that the development of the immune cells in the foetal liver is affected by the maternal physiological status.

Hypothesis 2 (Chapter 4): As all immune cells originate from the common progenitors, innate immune cell development may also be affected by the Hedgehog signalling pathway.

Hypothesis 3 (Chapter 5): Hh pathway plays an important role in NAFLD by altering the immune profile. To test this hypothesis, an adult model of diet induced obesity and NAFLD is used.

Hypothesis 4 (Chapter 6): Based on the findings from the above three hypotheses, this hypothesis investigates the interaction between the Hedgehog signalling pathway and maternal dietary status, which may be the missing puzzle of the developmental programming mechanism.

Chapter 2. Materials and Methods

2.1. Animal Handling

The colonies of Gli3+/- and Shh+/- mice were maintained and expanded in trios, by breeding two WT female C57/BL6 mice with one genetically modified male studs. All offspring were then ear biopsied and genotypes. Gli3+/- and Shh+/- female offspring were kept for further experiment. All mice were housed in pairs when possible.

To follow the 3Rs (Reduction, Refinement and Replacement), the numbers of animals used were calculated using the power calculation, to minimise the required number of animals, but still obtain statistically significant results (Tannenbaum and Bennett, 2015). All animals were housed in a group of two per cage and subjected to a 12-hour light/dark cycle. All animals were treated in accordance with the Animals (Scientific Procedures) Act. 1986 guidelines. Wild Type mice (WT, female C57BL/6), were purchased from Charles River Laboratories UK, Gli3+/- mice were purchased from Jackson Laboratories, and Shh+/- were a gift from P. Beachy (Chiang et al., 1996). All mice were on C57BL/6 background, maintained at University College London (U.K), and were genotyped by polymerase chain reaction (PCR). The feeding protocol was based on previous studies from our group, where it was shown to be an effective mouse model for obesity and NAFLD shown by liver histology (Samuelsson et al., 2008, Oben et al., 2010b). The mice were fed standard chow RMI (4% simple sugars, 3% fat, 50% polysaccharide, 14% protein [w/w], Special Dietary Services, energy 3.5 kcal/g) *ad libitum.* At around 10 weeks

old, half of each experimental group were switched to a commercial energy-rich, high palatable obesogenic diet 824053 (10% simple sugars, 23% fat, 28% polysaccharide, 23% protein [w/w], Special Dietary Services, energy 4.5 kcal/g) supplemented *ad libitum* with sweetened condensed milk (approximately 55% simple sugar, 8% fat, 8% protein, w/w, nestle) and supplemented with mineral mix (AIN93G, Special dietary Services, 125 mg/pot) **(Table 1).**

Dietary Constituents	Chow (RM1)	Obesogenic	Sweetened Condensed Milk
Protein	14.38%	23.0%	8.0%
Polysaccharides	50%	28.3%	0.0%
Simple sugars	4.05%	10.5%	56%
Fibre	4.65%	4.6%	0.0%
Lipid	2.71%	22.6%	9%
Energy (kcal/g)	3.52	4.54	3.25
Source	SDS 801151	SDS 824053	Nestle

Table 3: Dietary composition of control and obesogenic diet.

2.1.1. Time-mating

Adult female mice were fed with either a control diet or the experimental diet for eight weeks, they then entered the time-mating protocol, which involved placing a male adult mouse with two females and monitoring the females for vaginal plugs. The day the plug was found was designated Embryonic Day 0.5 (E 0.5). Mice failing to become pregnant after development of a vaginal plug were allowed to re-mate up to the age of 6 months **(Figure 7).**
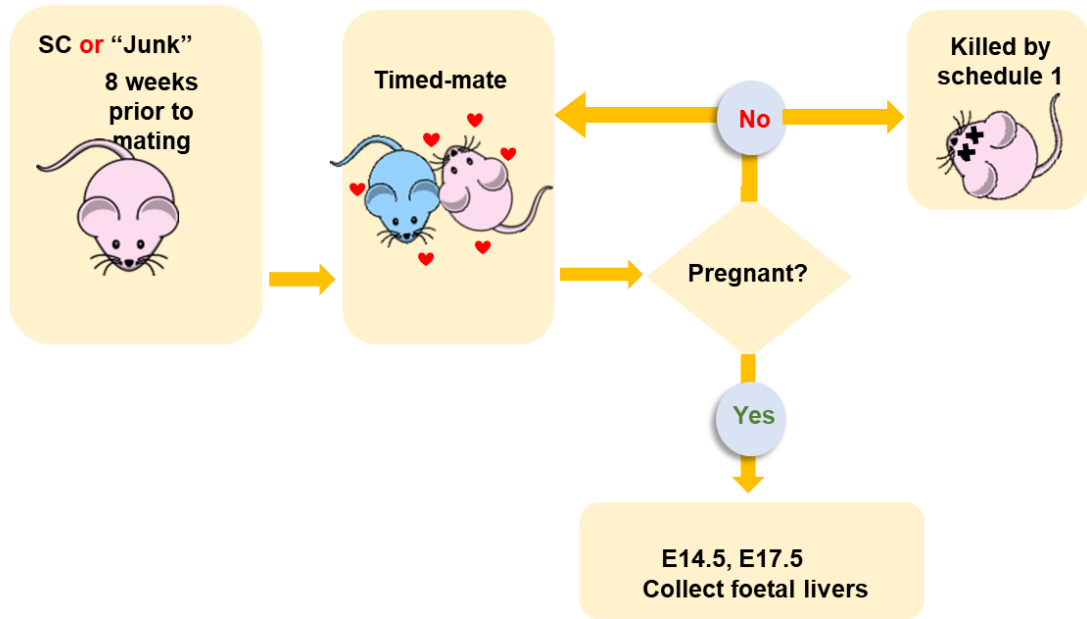
Figure 7. Time-mating protocol.

Female Gli3+/- and WT mice were fed with either control or high-fat/high-sugar diet for eight weeks before being time-mated wih a male WT mouse. At E14.5 and E17.5, the tissue was harvested.

2.1.2. Genotyping

Gli3+/- mice have an extra digit on one of their hind paws, so can be identified phenotypically in both embryos and adult by visualisation. All mutant mice were confirmed by genotyping **(Figure 8)**. The ear biopsies or tails of the embryos were digested in Proteinase K (concentration 1:100) at 56 °C with agitation (300rpm) for 4 hours, and then confirmed with PCR and gel electrophoresis on 1% gel. In adult mice, only tissue from ear punch was used in genotyping.

2.2. DNA extraction

DNA from mice extracted in 100mL lysis buffer (50mM KCL, 10mM Tris HCl (pH8.5), 1.5mM MgCl$_2$, 0.01% gelatin, 0.45% Noident P-40, 0.45% Tween 20) and 0.5µg/mL Proteinase K (Sigma-Aldrich) in RNAse free water (life Technologies) and incubating on a shaker at 900rpm at 56°C for five hours. The samples were then spun at 13000rpm in a micro-centrifuge for 5 minutes before use. 1µL DNA sample was then taken for 20µL of total mixture, 50% 2X GreenQag DNA polymerase and 1µL of each relevant primer, as described in Solanki et al (Solanki et al., 2018).

Gli3

Step	Temperature	Time
Denaturation	94°C	5 min
	94°C	1 min
	59°C	1 min 20s
	72°C	1 min
Final extension	72°C	10 min
Final hold	4°C	∞

Shh

Step	Temperature	Time
Denaturation	94°C	5 min
	94°C	40 s
	58°C	1 min
	72°C	40 s
Final extension	72°C	10 min
Final hold	4°C	∞

2.3. Tissue collection

2.3.1. Adults

The animals were fasted overnight and underwent glucose tolerance test. All adult animals were killed using Schedule 1 with rising CO_2. The mice perfused through the portal vein with saline. The liver was harvested in 5mL RPMI in 15 mL falcon tubes, and then divided into smaller pieces and preserved for further experiments: fresh in RPMI for immune cell isolation, fixed in 10% formalin for histology or snap frozen on dry ice for molecular analysis.

2.3.2. Foetuses

The WT and Gli3+/- mothers were terminated at E14.5 or E17.5, Shh+/-
mothers were terminated at E14.5 during the pregnancy with rising CO_2. The
embryos were then dissected from the amniotic sacs. Their body mass and
placenta mass were recorded. The livers, thymus and spleens of embryos
were harvested under the microscope.

2.4. Fasting Glucose tolerance test

This test is used to measure the clearance of an intraperitoneally injected
glucose load from the body, to be interpreted as indicators for diabetes or
metabolic syndrome. The fasting blood glucose levels are determined using a
glucometer (Accu-Check Aviva Blood Glucose System). The adult mice had
their food withdrawn overnight, for 12 hours. The mice were then injected with
a bolus of 200µl of 20% glucose solution (2g of glucose/kg body mass)
intraperitoneally. The tail of the mouse was scored using a sharp scalpel. The
first drop of blood was discarded, and readings were taken from the second
drop using the glucometer. The blood glucose levels were measured at 15, 30,
60 and 120 minutes after the injection.

2.5. Blood serum collection

Blood of the animals were collected via the inferior vena cava or the carotid artery into endotoxin-free Eppendorf vials. It was allowed to clot at room temperature for 15 to 30 minutes and then centrifuged at 1500g for 15 min at 4°C. The clear phase of the serum was then aliquoted to a new Eppendorf tube and stored at -20 °C until further analysis.

2.6. Cell counting

During this project, three different methods of cell counting have been used: traditional cell counting with trypan blue, NucleoCounter® and cell counting using Accuri C6.

2.7. Flow cytometry

2.7.1. Tissue preparation for adult livers

Approximately 1.5g of adult liver was cut into small pieces using a sharp scalpel, and then digested in 5mL of digestion medium (50µg/L of DNase I, 500ng/L of collagenase D) at 37°C for 30 minutes, the mixture was shaken half way. It was then filtered through a 70µm gauge, and centrifuged at 50g with no deceleration, at 4°C to eliminate dead hepatocytes.

2.7.2. Tissue preparation for foetal organs

Foetal livers were ground between two pieces of glasses to prepare cell suspensions in phosphate buffer saline (supplemented with 2% FCS and 0.01% sodium azide). The number of cells was counted, and the concentration is made up to 1×10^6/ml.

2.7.3. Staining

The cells were then stained with antibodies for 30 min on ice in a dark condition in 50 µL of phosphate buffer saline (supplemented with 2% FCS and 0.01% sodium azide), the following anti-mouse antibodies were used **(Table 2)**. Single stains were used for compensation. For hard to distinguish populations, FMO was used to show the gating of the population. The cells were then washed in DMEM, and run on a FACScan Accuri, or LSRII. All results were later analysed using FlowJo software. The dead cells are excluded using viability dye. Doublets are discriminated by FSC-H against FSC-A.

2.7.4. List of concentrations:

Antibody	Concentration	Source
CD3 FITC	1:100	Biolegend
CD4 FITC	1:100	Biolengend
CD 11B FITC	1:100	Biolegend
NK1.1 PE	1:100	eBioscience
CD11B PE-Cy7	1:200	eBioscience
F480 PE	1:100	eBioscience
F480 APC	1:200	
LY6G PE	1:100	eBioscience
GR-1 FITC	1:100	eBioscience

2.7.5. Compensation

There are overlaps between the spectrum emitted by the different stains, so to avoid false positives, so samples were compensated using the compensation algorithm from the FlowJo software.

2.8. RNA Extraction

The RNA from the foetal livers was extracted using a commercial kit: Absolutely Total RNA & mRNA Purify Kits (Agilent Technologies).

For adult tissue, RNA was extracted using the trizol method, where 1mL of Trizol ^{TM} was used per 10cm^2. **Homogenise and phase separation:** 25-50mg

of liver tissue was homogenised with autoclaved micropestles in 2mL Eppendorf tubes. The samples were then incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein. 300 µL of chloroform was then added to the sample and vortexed for 15 seconds. The samples were then rested at room temperature for 2 to 3 minutes. They were then centrifuged at 12000g, 15 min at 4°C, to allow phase separation. 500 µL of the supernatant is then taken from the sample and transferred to a fresh tube. **RNA precipitation:** 700 µL isopropyl (2-propanolol) was added to precipitate the RNA from the supernatant. The tubes were incubated at room temperature for 10 min, then centrifuged at 12000g for 10 min at 4°C. The mRNA would now be in the pellet at bottom of the tube. The supernatant was carefully removed without disturbing the pellet. 1.5mL of 75% ethanol was added to the pellet and vortexed to wash the RNA. The sample was then centrifuged at 7500g for 5 min at 4°C, and the supernatant was carefully removed. The pellet was then washed for the second time by adding 1.5mL of 75% ethanol, and centrifuged at 7500g for 5 min at 4°C. The supernatant was again discarded. The pellets were dried as much as possible with a small pipette. Lastly, the RNA was dissolved with 100 µL nuclease free water.

The quality and concentration of the RNA were analysed using Nanodrop ND-1000, A260/280 ratio and A260/230 ratio were recorded. Only good quality RNA, with an A260/280 ratio of between 1.8 to 2.1 was used. The

RNA contents were then reverse transcribed to cDNA, transcribed RNA was stored at -80°C.

2.9. Reverse-transcription polymerase chain reaction (RT-PCR)

The RT-PCR is a two-step process involving elimination of genomic DNA and then reverse transcribed in order to obtain complementary DNA (cDNA). This was performed using QuantiTect ® Reverse Transcription Kit (cat. Nos. 205310).

2.10. Polymerase chain reaction

Protocols follow the Minimal Information for Publication of Quantitative Real-time PCR Experiments (MIQUE) guidelines (Bustin et al., 2009). qPCR was performed using the QuantiTect SYBR Green PCR system. All cDNA samples were analysed by triplicates on an icycler (Bio-Rad laboratories, Hercules, CA). The results were analysed using $2^{-\Delta\Delta CT}$ algorithm. For the house-keeping gene, 18S and GAPDH were used as the controls.

2.11. Primer design

The primers to quantify the expression of the genes of interest were design using nucleotide base sequence from NCBI reference sequence database. The sequence was then analysed by Primer 3 software to ensure the optimum processes: that the primers anneal effectively to exon-intron-exon complexes or cross exon/exon boundaries, and the primers are not self-complementary or complementary to each other.

2.12. List of primers used

Gene	Primer sequence
IL-6	F: 5'-TCAATTCCAGAAACCGCTATG-3'
	R: 5'-GTCTCCTCTCCGGACTTGTG-3'
TNF-α	F: 5'-TCCAGCTGACTAAACATCCT-3'
	R: 5'-CCCTTCATCTTCCTCCTTAT-3'
ASMA	F: 5'-ATCTGGCACCACTCTTTCTA-3'
	R:5'-GTACGTCCAGAGGCATAGAG-3'
TGF-β	F:5'-AAAATCAAGTGTGGAGCAAC-3'
	R:5'-CCACGTGGAGTTTGTTATCT-3'
Collagen 1-α2	F:5'-GAACGGTCCACGATTGCATG-3'
	R:5'-GGCATGTTGCTAGGCACGAAG-3'
GAPDH	F: 5'-CGTCCCGTAGACAAAATGGT-3'
	R: 5'-TCAATGAAGGGGTCGTTGAT-3'

2.13. Statistical analysis

The data were analysed with Microsoft Excel and SPSS IBM 22.0, and the graphs were constructed using GraphPad Prism 7.0. The results were reported as mean+/- standard error of mean (SEM). The following statistical test were used: student t-test for data with two variables, Multi-T test with post-hoc analysis for continuous results, one-way ANOVA with post-hoc analysis and two-way ANOVA for result with more than two variables. $p < 0.05$ was considered statistically significant.

Chapter 3. Maternal obesity influences the immune profile of offspring liver *in utero*

3.1. Introduction

Non-alcoholic Fatty Liver Disease (NAFLD) is a serious liver condition, with the majority of cases caused by obesity, such that approximately 80% of the obese population have moderate to severe NAFLD, and the prevalence has increased rapidly in recent years (Bellentani et al., 2010). This fast rate of increase can be explained by the modern lifestyle to a certain extent: lack of exercise and energy dense food. Yet the phenomenon cannot be explained by these factors alone. Therefore, it has been proposed by many studies that the developmental programming (DP) effect may play a role in the rapid rise of this condition, such that this phenomenon underpins the 'first-hits' in line with the 'multi-hits' hypothesis of NAFLD pathogenesis (Stewart et al., 2013). Based on Barker's Hypothesis, that insults and stimuli at critical periods of development can influence health status, many studies have suggested that being subjected to maternal obesity at critical points of development leads to higher risk of developing NAFLD (Oben et al., 2010a). There is evidence from both human and animal studies, that offspring born from obese mice have higher body weight despite being fed a standard post-weaning diet. In this study, female mice were made to be obese by a high fat diet, modelling the human diet and were kept on the same diet during pregnancy and lactation. The adult offspring from these mothers were studied at 3 months, 6 months and 12 months, and the adult offspring born to the obese mothers displayed characteristics of NAFLD (Oben et al., 2010a). Research in this area was further expanded by utilising a cross-fostering model, to suggest that the lactation period is

especially important for maternal obesity to exert an effect on the phenotype of the offspring liver (Oben et al., 2010a). Furthermore, a more physiological mouse model has also shown that maternal obesity affects the phenotype of the offspring liver with hepatic immune alteration, marked by an increase in macrophages in the liver and reduction in natural killer T (NKT) cells (Mouralidarane et al., 2013).

Despite the strong evidence of a DP effect on NAFLD, the questions remain on how early the effects take place. In line with Barker's hypothesis, which is also known as the foetal origin of health and disease (Barker, 1998), it has been suggested that the maternal impact on NAFLD takes place early during the foetal stage, when organisms are at their most plastic and vulnerable. However, a limited number of studies have been conducted to investigate the maternal dietary effect on offspring livers during these early stages. One human study using magnetic resonance imaging (MRI) scans, observed that maternal obesity and gestational diabetes predict hepatic lipid storage in the foetal livers (Brumbaugh et al., 2013). Another study utilising a mouse model, traced the origin of developmental programming as early as the gametes level, as the oocytes and sperms from mothers and fathers with obesity were shown to have an increase in inflammatory profile (Huypens et al., 2016).

Here we study the influence of maternal obesity on development of the offspring liver *in utero* at E14.5 and E17.5. Similar studies have been

conducted to investigate the effects of maternal obesity during pregnancy, but the focus of these studies is on the well-being and characteristics of the mothers, rather than the foetuses (Musial et al., 2017). Only limited attention has been paid to the impact of perinatal obesity on the foetal livers and let alone studying the influence of maternal obesity on the immune environment of the foetal livers at such an early stage. Due to the long latency and certain limitations of human studies, we turn to animal models to observe this phenomenon, with rodents as the choice of experimental subjects.

Aim: the aim of this chapter is to build on our previously established feeding and breeding model (Oben et al., 2010a), to study the offspring liver at a much earlier stage of development, under the influence of peri-natal obesity.

3.2. Method

10 weeks old adult wild-type (WT) female mice were subjected to either control (Con) or high fat/high-sugar (Ob) diet for 8 weeks and were then time-mated with a WT male stud. The presence of a vaginal plug in the morning was counted as embryonic 0.5 (E0.5). The foetuses were harvested at E14.5 and E17.5. The characteristics of the mothers were recorded, and the weight of the foetuses, placentas and the whole liver cell numbers were recorded. All foetal livers were analysed for their immune profile by flow cytometry and stained for NK cells (NK1.1), Macrophages (F4/80, CD11B), granulocytes (GR1, LY6G),

B cells (B220, CD19) and T cells (CD3, CD4, CD8) using Accuri C6 flow cytometer. The transcriptomic changes were analysed by real-time polymerase chain reaction for the following genes: IL6, TNF-α, Col1α, αSMA, TGFß, and Gli3 (Solanki et al., 2017). IL6, TNF-α were used as markers of inflammation. Col1α, αSMA and TGFß were used as markers of fibrosis. TGFß and Gli3 were used to observe developmental effects.

3.3. Results

3.3.1 Maternal characteristics: the gestational weight gain for the obese mothers is significantly higher than the control group at E17.5, but not at E14.5.

As mice age, they naturally gain some weight from their baseline, but the mice fed with the high fat diet (HFD) gained weight much more rapidly compared to the control mice **(Figure 8A)**. By the end of the eight weeks, which was also the time point these mice entered into the time-mating protocol, these mice on the HFD had significantly higher body weight compared to the control, such that all female mice fed onto HFD gained more than 30% of their baseline weight **(Figure 8B)**. This served as a good model representing the human populations, where many women are obese before they became pregnant, and consumption of high fat and high sugar diet is common during pregnancy. The higher body weight in the obese group persisted throughout the pregnancy, such that at E14.5, there remained a significant difference **(Figure 8C and D)**.

However, the gestational gain at E14.5 was not different between the two groups **(Figure 8 E)**. This implies that although at baseline pre-pregnancy stage the obese mothers were heavier over the span of 14 days, they gained a similar amount of weight as the control group.

Similarly, at E17.5, the maternal weight of mice fed on HFD was higher than the maternal weight of the control mice **(Figure 8 D)**. Yet, in contrast to E14.5 gestational weight gain for E17.5 group was also significantly higher in the obese group compared to the control group **(Figure 8 E)**. This suggests that the major changes occurred between E14.5 to E17.5. Additionally, it is interesting to note, that the mothers on the obese diet had a lower fertility rate, which was calculated as the percentage of vaginal plugs formed within four days of entering the time-mating protocol (four days was used because the oestrus cycle of a mouse is around four days) **(Table 4)**. The obese mothers also had lower pregnancy success rate, which was calculated as the percentage of successful pregnancies after a vaginal plug was found **(Table 4)**. Furthermore, the obese mothers were also more likely to have resorbed foetuses than the control group.

	Control	Obese
Fertility rate %	93.6	69.2
Pregnancies %	75.6	42.3

Table 4: The obese mothers had lower fertility rate and lower chance of successful pregnancies.

3.3.2 Foetal characteristics at E14.5 and E17.5

3.3.2.1 The foetuses of obese mothers have lower body weight compared to the foetuses of control mothers at E17.5.

At E17.5, it was observed that the foetuses from the obese mothers were significantly smaller in weight compared to the foetuses from the control mothers, with a mean weight of 0.63±0.03 and 0.73±0.03 ($p < 0.05$) respectively **(Figure 9 A)**. However, the weights of the placentas were not different between the control group and obese group **(Figure 9 B)**. The ratio between foetus weight and placenta weight, denoted as FW:PW, is often used as an indicator of the efficiency of the nutrient exchange between the mothers and the foetuses (Wilson and Ford, 2001). Yet, at E17.5, despite the difference in the weight of foetuses and lack of differences in placental weight, there was no observed difference in the foetus/placenta ratio between the two groups **(Figure 9 C)**. However, for the number of liver cells, the foetal livers from the obese mothers contained significantly fewer cells compared to the control group **(Figure 9 D)**.

3.3.2.2 There are no differences in the foetal weight between the obese group and the control group at E14.5

At E14.5, there were no differences between the weight of the foetuses in the two experimental groups **(Figure 10 A)**. The placentas are also similar in size, and the ratios between the foetuses and the placentas did not show a difference **(Figure10 B and C)**. However, the foetal livers from the foetuses of the obese mothers exhibited a significantly lower number of cells compared to the foetal livers of the control group **(Figure 10 D)**. This indicates that although there are no obvious weight differences, changes have been induced by the maternal dietary status. Additionally, the fact that at E14.5, the weight of the foetuses remains the same, yet at E17.5, the weight of the foetuses was difference, indicates that changes have occurred during this 3-day window.

3.3.3 The immune profile of foetuses at E17.5

3.3.3.1 Maternal obesity alters the innate immune profile of the foetal livers at E17.5

Macrophages in the foetal liver were identified using F4/80 and CD11B markers. In this study, there was no difference in the percentage of macrophages between the two experimental groups, suggesting that the

macrophage population was not affected by the dietary status of the mother **(Figure 11 A and B)**. However, the NK cell population, which was identified as CD3$^-$NK1.1$^+$, showed a significantly higher percentage in the livers of foetuses born to the obese mothers compared to the livers of foetuses born to the control mothers **(Figure 11C and D)**. NKT cells, which were identified by CD3$^+$NK1.1$^+$, were not detected by this flow cytometry analysis, so no comparison can be made for this cell group **(Figure 11 C and D)**.

For the granulocytes, the percentage of the GR1$^+$ population was higher from the livers of the foetuses from the obese mothers compared to the control mothers **(Figure 12 A)**. To further differentiate the granulocyte population, the marker Ly6G was also used to identify neutrophils and eosinophils. There was no difference in the proportion of the neutrophil (GR1$^+$Ly6G$^+$) population between the two experimental groups **(Figure 12C and D)**. However, the eosinophil population, GR1+Ly6G-, was higher in livers of foetuses born to obese mothers compared to the livers of foetuses born to the control mothers **(Figure 12 E)**.

3.3.3.2 Maternal obesity alters the adaptive immune profile of the foetal livers at E17.5

At E17.5, the B-cell population was also altered by the maternal dietary status. There was a higher percentage of CD19$^+$ cells in the livers of the foetuses born to the obese mothers compared to the control mothers **(Figure 13)**.

3.3.4 The immune profile of foetal livers at E14.5

3.3.3.1 Maternal obesity alters the macrophage population and NK cell population at E14.5

At E14.5, the macrophage population was affected by the maternal dietary status **(Figure 14 A and B)**. It was found that the livers from foetuses born to the obese mothers had a lower percentage of macrophages compared to the foetuses born to the control mothers **(Figure 14 A and B)**. Similarly, the NK cell population was also reduced, such that there was a lower percentage of NK cells in the livers of foetuses born to the obese mothers compared to the control mothers. The marker CD3 was used in conjunction with NK1.1 to identify the NK1.1+CD3+ NKT cell population but the NK1.1-CD3+ T cell population was not detected. The NK1.1-CD3+ cell population was significantly reduced in the obese group compared to the control group, indicating that the T cell lineage was reduced **(Figure 14 E)**.

3.3.3.2 Maternal obesity alters the GR1+ population, yet there appears to be no difference in neutrophils and oesinophils

At E14.5, there was a significant difference in the percentage the GR1+ population, such that there were less GR1+ cells in livers of foetuses born to the obese mothers, compared to the control mothers **(Figure 15 A)**. However, when the scatter diagram was analysed for GR1 and LY6G, there appeared to be no significant difference between the two groups **(Figure 15 C and E)**.

3.3.3.3 Maternal obesity affected the B cell population at E14.5

The percentage of B220+CD19+ B cells was significantly reduced in the obese mother group compared to control **(Figure 16 A and B)**, indicating that the obese diet influenced B cell development. However, there were no significant differences in the proportions of the progenitor B220+C19- or B220-C19+ populations **(Figure 16 B and C)**

3.3.5. Foetal gene expression at E17.5

The whole foetal livers from the two experimental groups were analysed for gene expressions by qPCR of inflammatory markers: *IL6* and *TNFα*; fibrogenic factors: *Col1-α*, *αSMA* and *Tgfß*; and the developmentally important gene: *Gli3*. At E17.5, there was a significant increase in the level of Tgf*ß* in the obese group compared to control **(Figure 17)**. Tgfß is not only involved in fibrogenesis, but also in many important signalling pathways，such as the hedgehog signalling pathway. The expression of *Gli3*, which codes a Hh-responsive transcription factor which represses Shh expression in the foetal of liver (Solanki et al., 2017), showed a significant increase in the obese group compared to the control group **(Figure 17)**. There were no significant changes for *IL6, TNF-α, Col1-α* and *αSMA.*
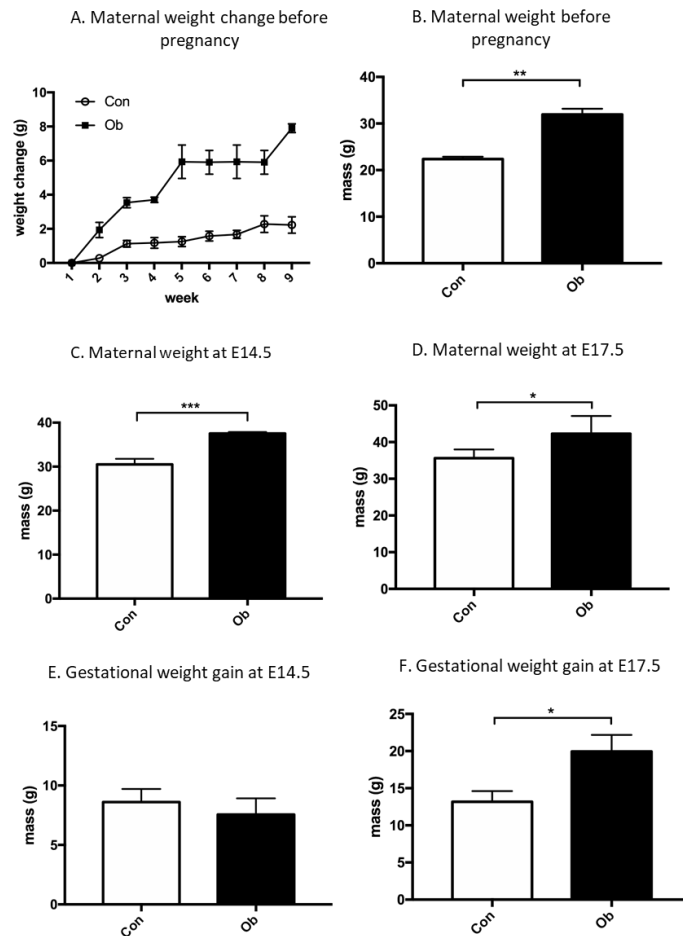
	Con	Ob	p-value
Whole body weight pre-pregnancy (g)	22.37±0.51	31.94±1.24	<0.01
Whole body weight at dissection E14.5 (g)	30.50±0.73	37.53±0.20	<0.001
Gestational weight gain E14.5 (g)	8.60±1.102	7.55±1.368	0.60
Whole body weight at dissection E17.5 (g)	35.6±1.18	42.27±2.17	0.04
Gestational weight gain E17.5 (g)	13.18±1.43	19.95±2.23	<0.05

Figure 8. Evolution of maternal weight before and during pregnancy.

(A) Weekly weight gain of both control and obese mothers during eight weeks of their respective diet; (B) bar chart showing the weight of the control and obese mothers immediately before pregnancy; (C) bar chart showing maternal weight at E14.5; (D) bar chart showing maternal weight at E17.5; (E) bar chart showing gestational weight gain at E14.5; (F) bar chart showing gestational weight gain at E17.5 compared to baseline E0. n=15, *p<0.05, **p<0.01, ***p<0.001

A. Foetal weight at E17.5

B. Placental weight at E17.5

	Con	Ob	p-value
foetal weight (g)	0.73±0.03	0.63±0.02	0.03
placental weight (g)	0.14±0.01	0.13±0.01	0.12

C. Foetus-placenta ratio

D. Foetal liver cell number at E17.5.

	Con	Ob	p-value
foetus/placenta ratio	4.76±0.31	5.26±0.41	0.53
Liver cell number	24166933 ± 2129110	10943000 ± 1587327	<0.001
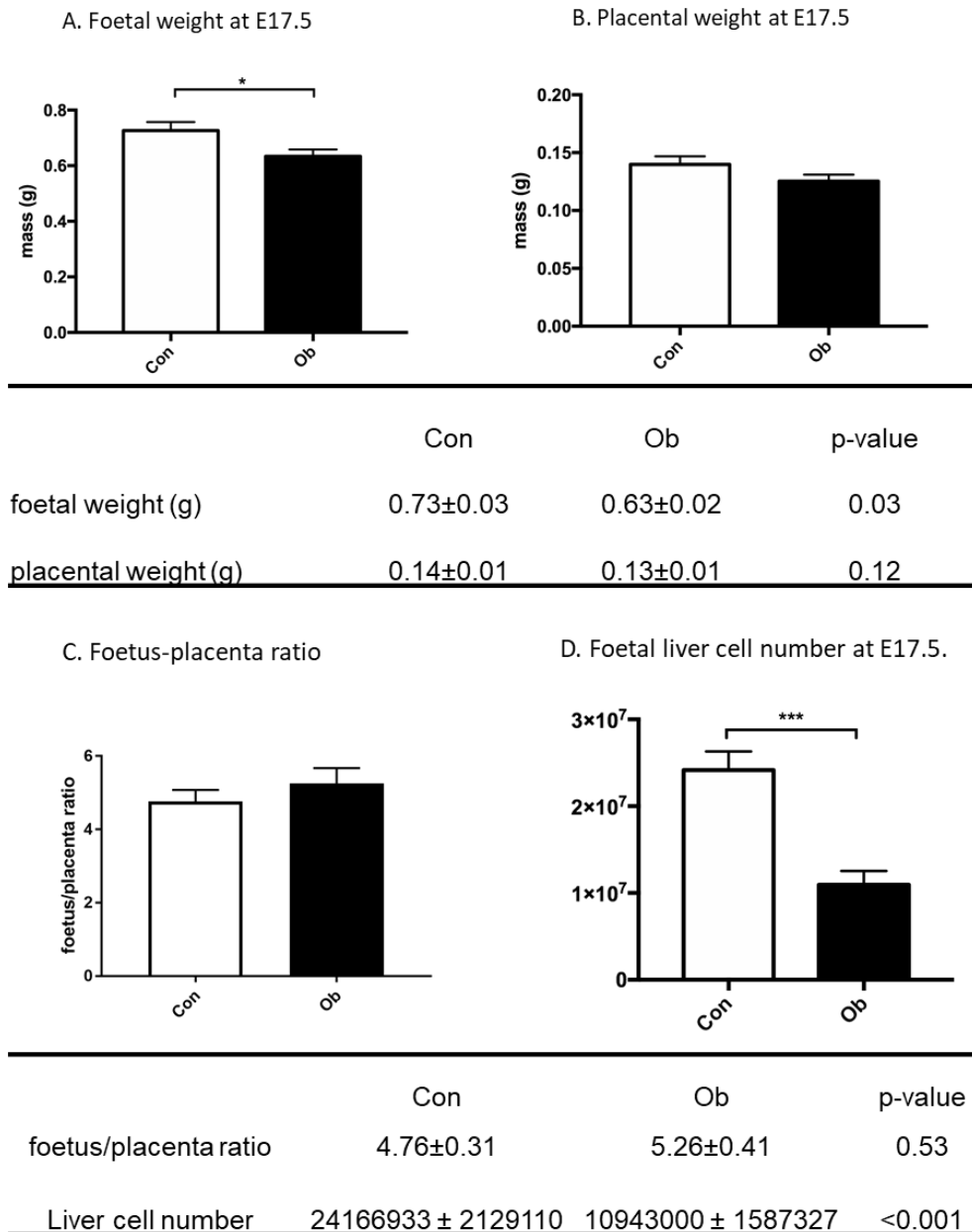
Figure 9. Characteristics of the foetuses at E17.5

(A) Bar chart showing the weight of the foetuses from two experimental groups; (B) bar chart showing the placental weight of the two experimental groups. (C) bar chart showing the foetus/placenta ratios of the two experimental groups. (D) bar chart showing the number of liver cells of the foetuses from two experimental groups. n=13, *p<0.05, **p<0.01

A. Foetal weight at E14.5

B. Placental weight at E14.5

	Con	Ob	p-value
foetal weight (g)	0.226 ± 0.00499	0.229 ± 0.00458	0.663
placental weight (g)	0.11 ± 0.00779	0.12 ± 0.00471	0.268

C. Foetus-placenta ratio

D. Foetal liver cell number at E14.5.

	Con	Ob	p-value
Foetus/placenta ratio	2.123±0.15	1.848±0.05	0.099
Liver cell number	2211292 ± 714793	614173 ± 106512	<0.001
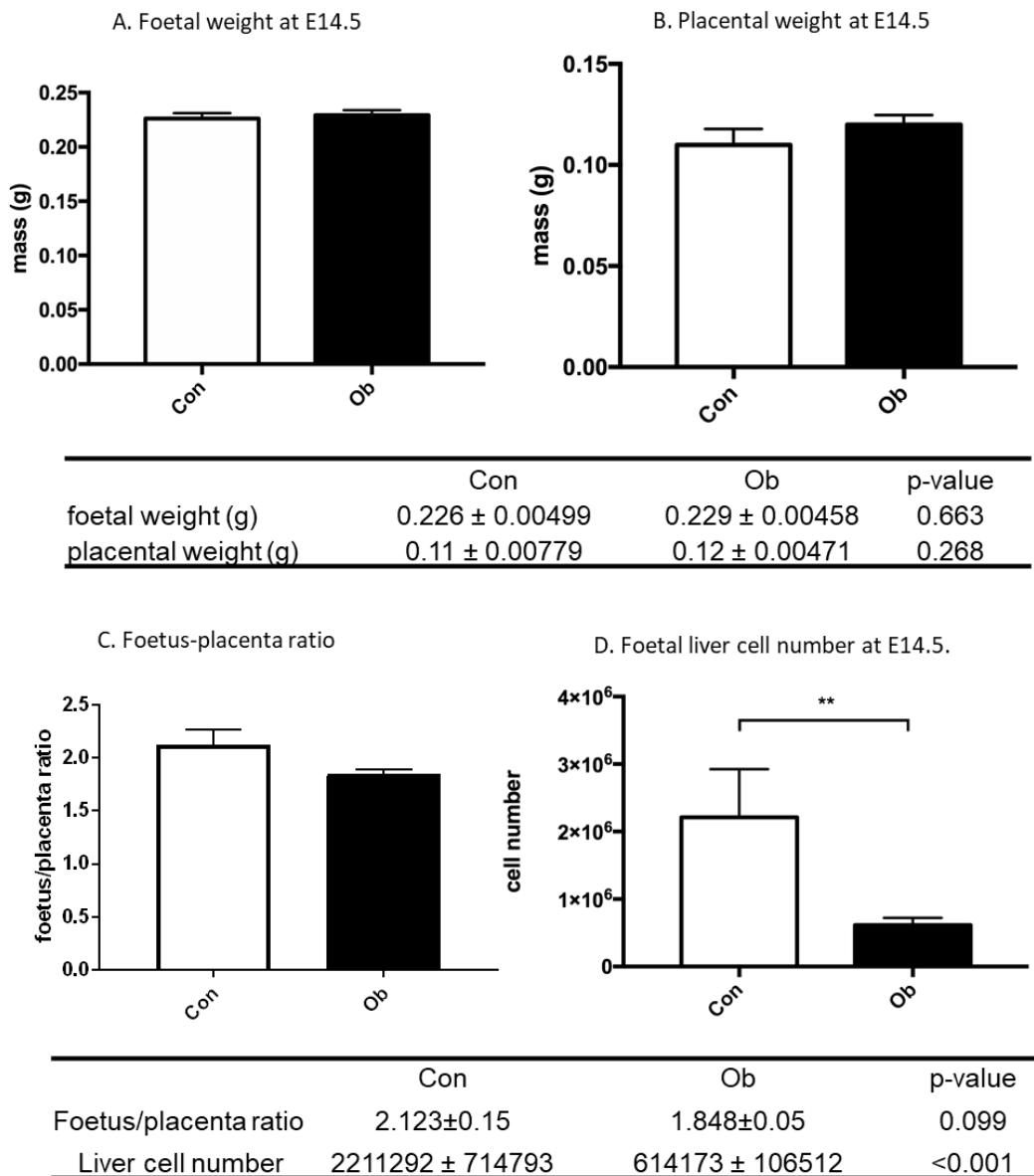
Figure 10: Foetal characteristics at E14.5.

(A) Bar chart showing the weight of foetuses from two experimental groups;

(B) bar chart showing the placental weight from the two experimental groups.

(C) bar chart showing the foetus/placenta ratios from the two experiment

groups. (D) bar chart showing the number of liver cells of fetuses from two

experimental groups. n=13, $*p<0.05$, $**p<0.01$, $***p<0.001$
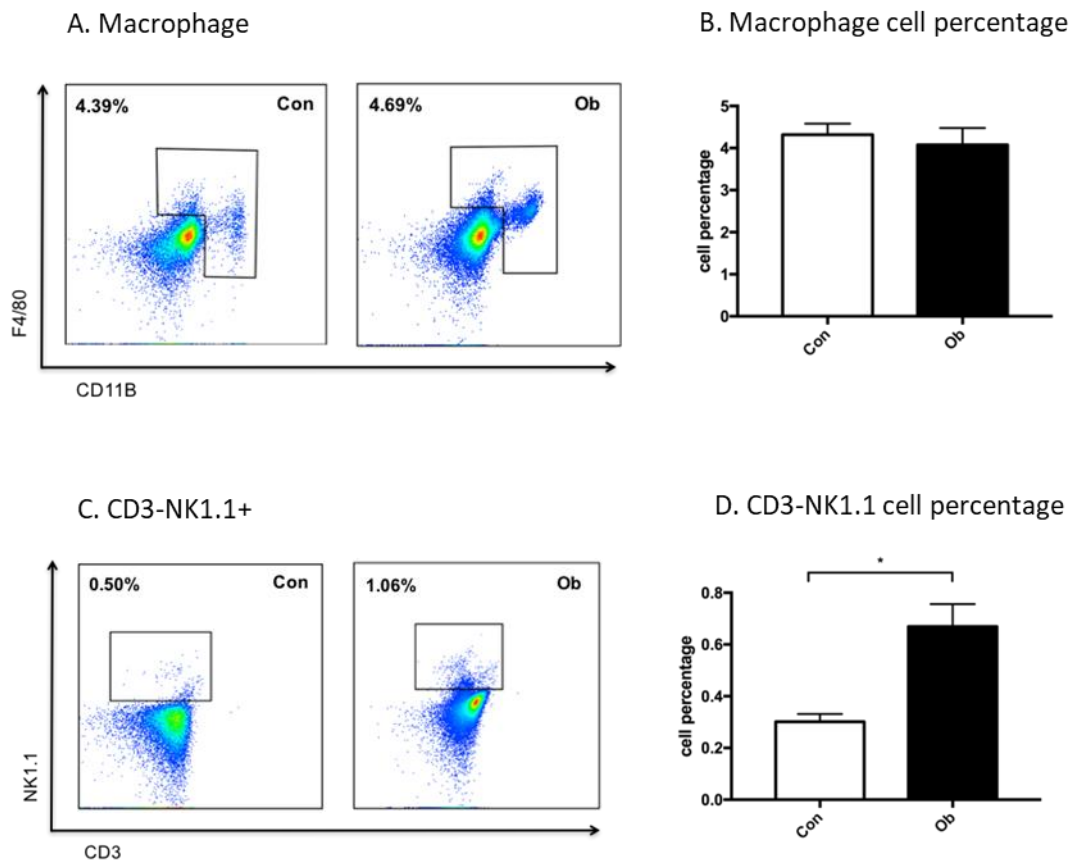
Figure 11: Macrophage profile of the foetal livers at E17.5.

(A) Flow cytometry diagram showing the macrophage population; (B) bar chart showing mean±SEM percentage of macrophage population for the two experimental groups (C) flow cytometry diagram showing NK cell populations; (D) bar chart showing mean±SEM percentage of NK cells from the two experimental groups. (E) bar chart showing mean mean±SEM percentage of T cells in the livers of foetuses from two experimental groups. n=13, *p<0.05.
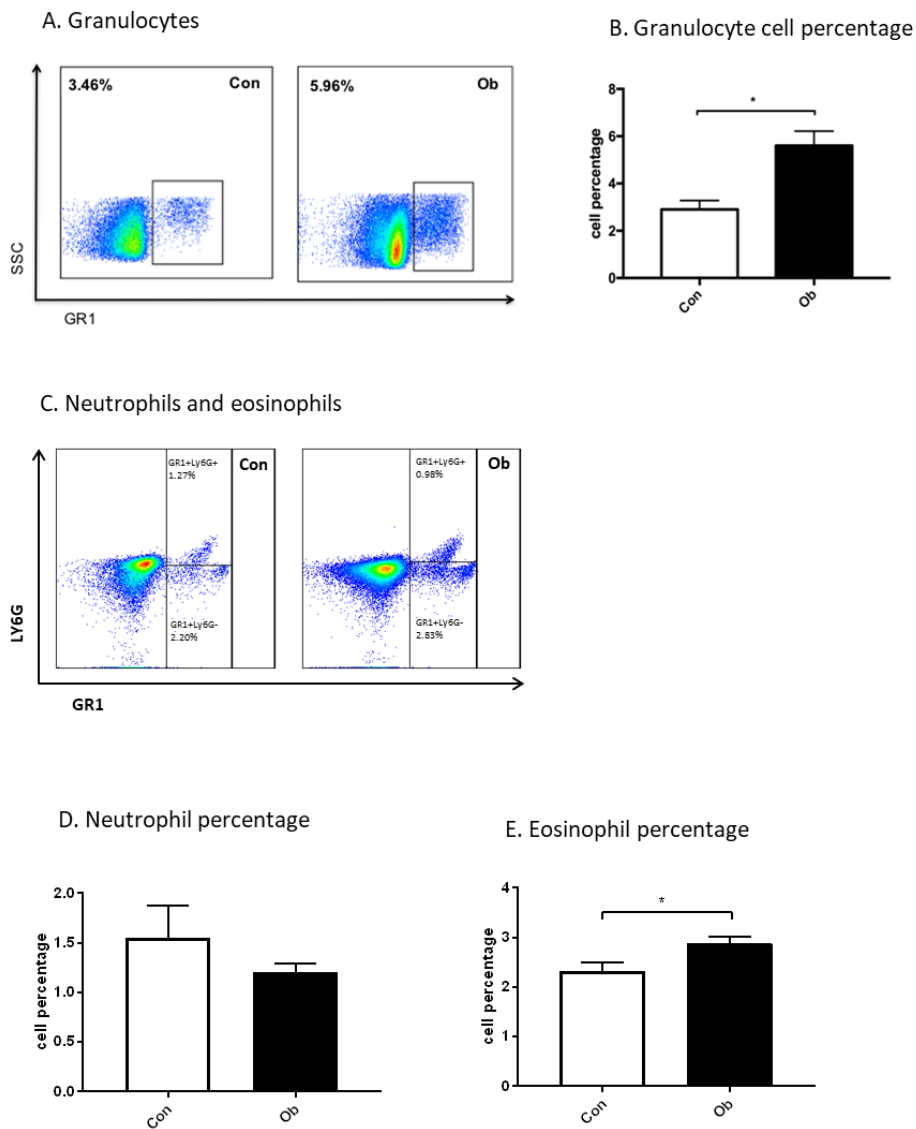
Figure 12: Granulocyte profile of foetal livers at E17.5:

(A) flow cytometry diagram of the granulocytes from two groups; (B) bar chart showing mean±SEM percentage of granulocyte from the two experimental groups; (C) flow cytometry diagram showing the neutrophil and eosinophil populations; (D) bar chart showing mean±SEM percentage of neutrophils; (E) bar chart showing mean±SEM percentage of eosinophils from the livers of foetuses from two experimental groups. n=13, *p<0.05.
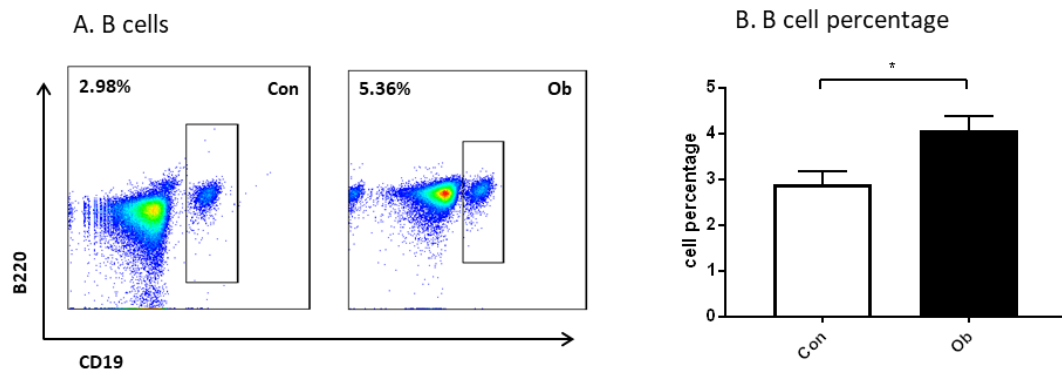
Figure 13: Maternal obesity alters the B cell population.

(A) flow cytometry profile showing the CD19+ population from the two experimental groups; (B) bar chart showing the mean±SEM percentage of CD19+ B cells of foetuses from two experimental groups. n=13, *p<0.05

Figure 14: **Macrophage profile of foetal livers at E14.5.**

(A) flow cytometry profile showing macrophages (F4/80+/CD11B+) from the two experimental groups; (B) bar chart showing mean±SEM percentage of macrophage population from two experimental groups; (C) flow cytometry profile showing NK cell population and T cell population; (D) bar chart showing mean±SEM percentage of NK cells population in livers of foetuses from two experimental groups; (E) bar chart showing mean±SEM of T cell in livers of foetuses from two experimental groups. n=13, *p<0.05, **p<0.01.
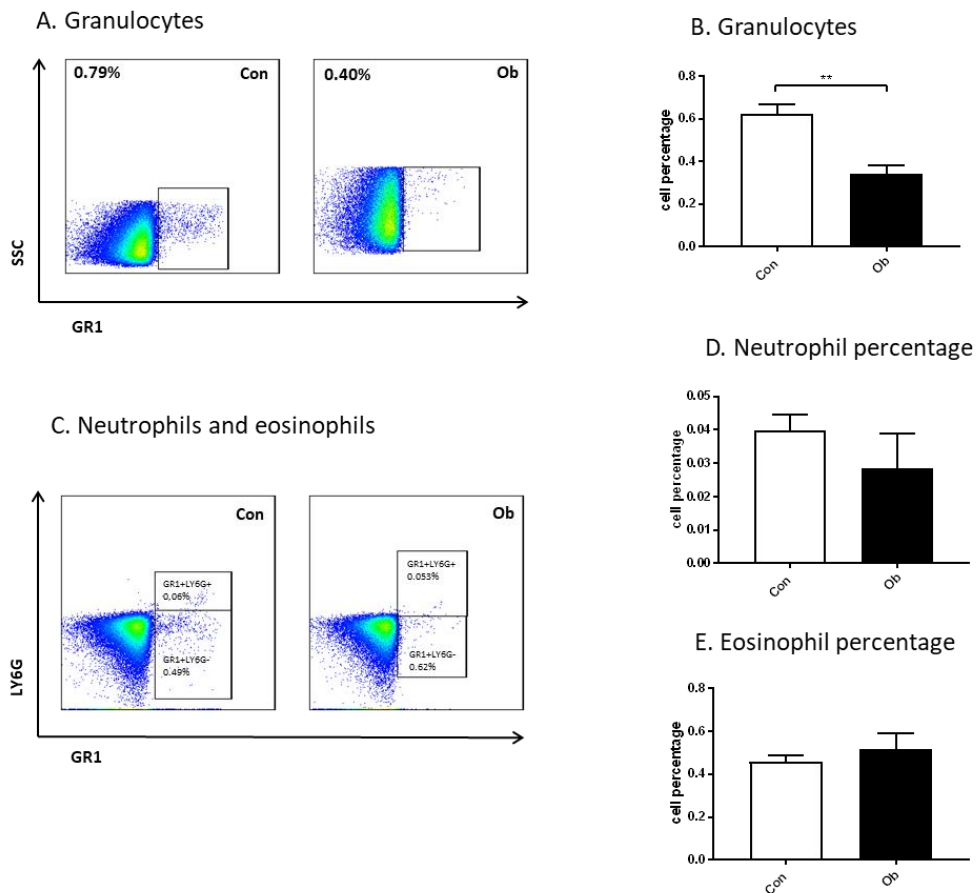
Figure 15: Granulocyte profile of foetal liver at E14.5.

(A) flow cytometry profile showing GR1+ population in control and obese groups; (B) bar chart showing mean±SEM percentage of GR1+ cells in livers of foetuses from two experimental groups; (C) flow cytometry profile showing neutrophil (GR1+LY6G+) and eosinophil (GR1+LY6G-) populations in foetal livers of two experimental groups; (D) bar chart showing mean±SEM percentage of neutrophils from two experimental groups; (E) bar chart showing mean±SEM percentage of eosinophils from two experimental groups. n=13, *p<0.05, **p<0.01

A. CD19/B220
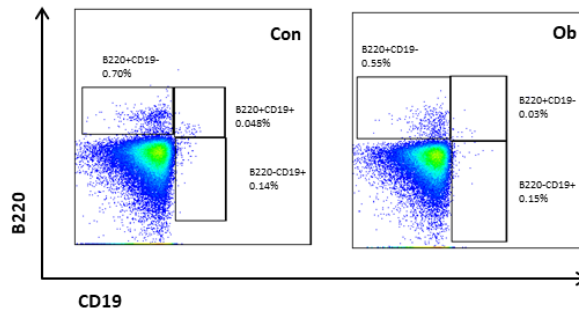
B. CD19-B220+ cell percentage
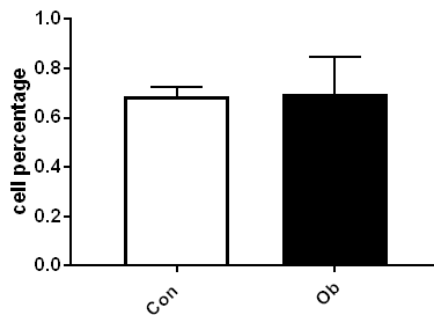
C. CD19+B220- cell percentage
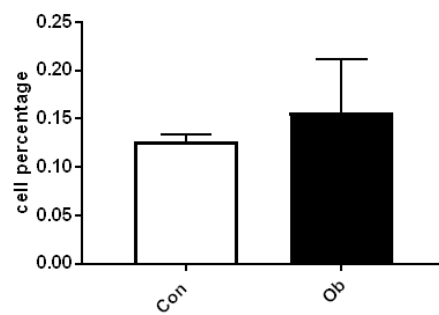
D. CD19+B220+ cell percentage
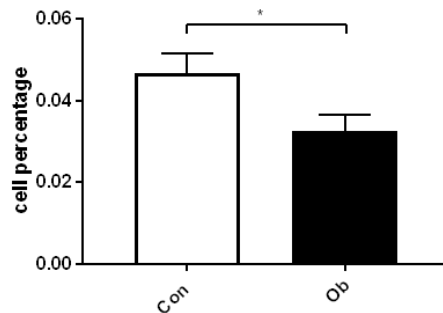
Figure 16: **B cell profile of foetal livers at E14.5.**

(A) flow cytometry profile showing B cell populations in control and obese groups;

(B) bar chart showing mean±SEM percentage of CD19-B220+ cell population;

(C) bar chart showing mean±SEM percentage of CD19+B220- cell population;

(D) bar chart showing mean±SEM percentage of CD19+B220+ cells population.

n=13, *p<0.05.
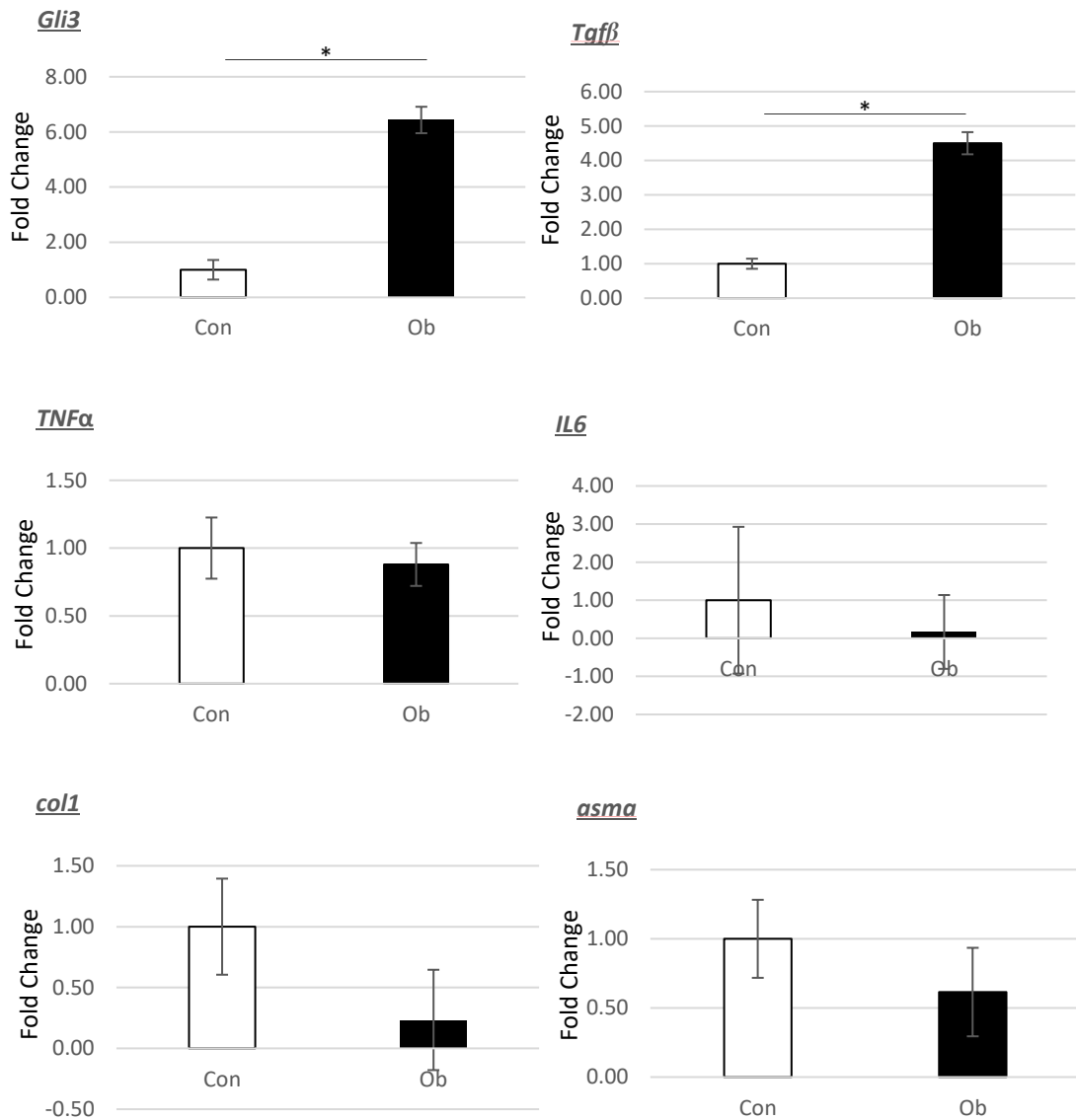
Figure 17: Gene expression of foetal livers from the two experiment groups.

Inflammatory markers: *TNFα, IL6*; fibrogenic markers: *Tgfß, Col1α, αSMA, Gli3.* n=5, *p<0.05.

3.3. Discussion

Early time points of development are critical windows for modelling the metabolic system, immune system and the epigenetics. This study shows that perinatal maternal obesity programmes the offspring as early as the intrauterine stage, such that the foetuses from the obese mothers had lower body mass than the foetuses from the control mothers at E17.5, a time point 3 days before birth. This is contrary to findings in humans, for which the foetuses from obese mothers tend to be large for gestational age (Kim et al., 2014). This could be due to the different developmental timeline of both humans and mouse embryos. Additionally, it is important to take into consideration that although rodents are used to model humans, fundamentally, they have very different physiological makeup and the third trimester of humans is equivalent to the weaning period of the mice (Dutta and Sengupta, 2016). The stunting effect on foetal growth by maternal obesity has been report by similar studies (Kamimae-Lanning et al., 2015). The only source of nutrients to the developing foetuses come from the foetal/placental interphase. Therefore, it is possible that the observed effect was due to placenta inefficiency. There is evidence that maternal obesity alters the structure and function of the placenta (Burton et al., 2016).

The ratio between the foetus weight and placentas weight (FW:PW) is often used as a reliable indication for efficiency of nutrient exchange between

mothers and the foetuses (Hayward et al., 2016). Placentas can act in an adaptive fashion, to accommodate the growth of the foetuses, such that the size of the placenta often dictates the size of the foetus. Yet, in this study, despite the observed difference in the foetal mass between the control group and the obese group, there was no observed difference in the placenta mass, and no difference in the ratio of FW:PW, either at E14.5 and E17.5.

Furthermore, it was interesting to observe that the major change in maternal weight occurred between E14.5 to E17.5. Unfortunately, in this study, it was difficult to record the food consumption of the mice. Therefore, it would be interesting to see whether the rapid weight gain was due to increased food consumption or decreased metabolism. The major change in weight between E14.5 and E17.5 is also consistent with the findings that the last trimester is when foetal growth is accelerated (Lampl and Jeanty, 2003).

For the foetal livers, it was found that the livers from the foetuses from the obese mothers contained fewer cells at both E17.5 and E14.5, indicating the foetuses from the obese mothers have smaller livers. This observation was also accompanied by a change in immune profile. Based on the previous study from our group, where it was observed that maternal obesity programs the offspring for NAFLD by affecting the macrophage and NKT cell populations, we investigated the macrophage and NK cell populations in this intrauterine study. However, contrary to the results in adults, at E17.5, there was no

difference for the macrophage population for foetuses from obese mothers compared to the control mothers. This indicates that the difference observed in our previous study may have taken place post-natally. In this study, NKT cells were not stained clearly. However, the NK cell populations showed an elevation in percentage in the obese group compared to the control group at E17.5. However, at E14.5, the NK cell population in the foetal livers subjected to maternal obesity seems to be depleted. It is known that NK cells have an intimate relationship with the pathogenesis of NAFLD, with studies showing different results, some correlating depletion of NK cells to NAFLD, and others associate depletion of NK cells to NAFLD (Tian et al., 2013).

The granulocytes followed a similar trend to the NK cells. There appeared to be more granulocytes in livers of foetuses born to obese mothers compared to the lean mothers at E17.5, yet at E14.5 the obese group had fewer granulocytes compared to the control group. At E17.5, the percentage of eosinophils was increased by maternal obesity, but the neutrophil population was unaffected. Eosinophils are often associated with worms and parasite infections (Furuta et al., 2014). One of the prevailing theories underlying the pathogenesis of obesity and NAFLD is the involvement of gut microbiota. There is a possibility that at this stage of the pregnancy, the immune system of the foetuses was affected by the microbiota changes in the mothers, which could very possibly be induced by the high-fat, high-sugar diet. The neutrophil and eosinophil populations in the foetal livers were unaffected at E14.5,

suggesting that time point may be too early to see any major alteration of the immune cell development.

Previously, our focus has always been on the modulation of the innate immune system. In this study, we also examined the lymphocyte populations in the foetal livers. At E17.5, the B cell population in the liver was higher from the foetuses of obese mothers compared to the control group. Whereas at E14.5, B cell population showed no differences between foetal livers of the control group compared to the obese group. These results suggest that the major changes in the immune system might have taken place at the third trimester of pregnancy.

To further explore the effects of maternal obesity on the offspring *in utero*, the following gene expressions were studied*: IL6, TNF-α, Col1α, αSMA, Tgfß* and *Gli3* in the mouse model. The inflammatory and fibrogenic markers did not seem to be affected by maternal obesity at this stage. This may be explained by the fact that NAFLD has always been a later life health condition, such that the early stages of development are not seen to be long enough to allow the inflammatory and/or fibrogenic effects to manifest. However, this does not mean no changes have taken place. Interestingly, the two genes which give elevated expression, are *TGFß* and *Gli3*, suggesting that developmental pathways are involved. Furthermore, *Gli3* gene serves as a repressor gene for

the Hh pathway, and can repress Shh expression in the mouse foetal liver (Solanki et al., 2017)

The findings from this study are novel, yet the results need to be analysed and interpreted with caution. The immune mechanism of NAFLD in adult humans and animals may act by different mechanisms from this foetal study. At E14.5 and E17.5, the foetal livers are the major organ responsible for haematopoiesis before the development of the immune cells moves to the bone marrow. This is important, as the altered effects from maternal obesity may be more systemic rather than just localised to the liver only. Consistent with the findings in this study, another study also showed that the chronic maternal obesity compromises the development of haematopoietic stem cells, marked by a decrease in haemopoietic progenitor cells. However, the same study also suggests that acute maternal obesity tends to boost the growth of immune cells (Kamimae-Lanning et al., 2015).

Foetuses are vulnerable due to the lack of buffering effect. Foetal subcutaneous adipogenesis progresses through gestation, yet lipid storage remains dormant until the third trimester, such that the subcutaneous fat is unable to buffer any excess glucose or fatty acids (McCurdy et al., 2009). This means that for the first two thirds of gestation; the energy is stored in the liver. Indeed, a study utilising a Japanese macaque NHP model, shows that consumption of a HFD during pregnancy reduces uterine blood flow,

increasing placental inflammation, such that foetuses at the third trimester already have hepatic steatosis (McCurdy et al., 2009).

3.4. Conclusion

Foetuses were affected by maternal obesity prenatally with an unexpected reduction in body mass at E17.5. This was accompanied by changes to the immune system. There was an alteration of granulocytes and NK cell population in the foetal livers. However, there was no observed alteration in the macrophage profile in the foetal livers, suggesting that the alteration and involvement of macrophages in NAFLD may come at a later stage of development.

The findings from this study are in line with the pathogenesis of NAFLD and suggest that maternal obesity impacts on the development of the foetal liver, causing the immune system in the foetal livers not to develop properly. The integrity of the foetal livers may be compromised by maternal obesity, leading the livers to become more vulnerable to future insults. This is especially the case in humans, where the offspring would be most likely to consume the same diet as their parents, and the combination of the two are most likely the clue for the rapid rise of NAFLD in the general population. Lastly, there was a difference between E14.5 and E17.5, and it would be interesting to investigate this further.

Chapter 4: The Hedgehog signalling pathway modulates the development of innate immune cells in the liver.

4.1 Introduction

The Hedgehog signalling (Hh) pathway is a key regulator of animal development and is involved in establishing the body plan (Pospisilik et al., 2010b). There are three types of mammalian Hh proteins: Desert Hedgehog (Dhh), Indian Hedgehog (Ihh) and Sonic Hedgehog (Shh), with Shh as the most well studied pathway. It is known that mice lacking both alleles of Shh (Shh knock out (KO), fail to develop organs properly and are embryonically lethal (Chiang et al., 1996). Besides being closely associated with early development, this pathway is also often associated with injury, repair and cancer (Zavros, 2008).

Hh plays an important role in foetal liver development. It has been shown that Hh signal activation coordinates the proliferation and differentiation of foetal liver progenitor cells (Hirose et al., 2009). It has also been reported that Shh ligands are expressed in the ventral foregut endoderm, where the liver derives, but the signal is lost at Embryonic Day (E) 9.0 to 9.5. In fact, observations from homeobox Hex knock out (KO) mice have shown that *Shh* expression needs to be shut off in order for normal liver development (Bort et al., 2006).

There are several studies which investigate the role of Hh in shaping the immune system, with many studies focusing on the involvement of Hh in lymphocyte development. Dependent on the stage of development the

pathway has been shown to promote and negatively regulate lymphocyte development (Crompton et al., 2007). Less research has investigated the role Hh in the development of the innate immune system. As all immune cells stem from the same progenitor cells, the effect of Hh on the lymphoid lineage may also suggest an effect of Hh on the myeloid lineage. One study utilising homozygous deletion of Gli1 revealed a decrease in myeloid development (Merchant et al., 2010). Other studies have highlighted that Hh impacts on the differentiation and function of innate-like lymphocytes (Kling and Blumenthal, 2017). Evidence from our previous studies and other existing studies suggest that the alteration of the innate immune system is involved in the pathogenesis of NAFLD, with involvement particularly of macrophages, NK cells and NKT cells (Mouralidarane et al., 2013). Syn et al also showed that NKT cells are responsive Hh signalling and produce Hh proteins (Syn et al., 2012). Therefore, here we utilise an *in-utero* model, to investigate the question to what extent Hh exerts influence on the innate immune cells, which are involved in the pathogenesis of NAFLD.

We used two constitutive mouse mutants to investigate the impact of Hh pathway on immune development in the foetal liver: the Shh-/- and Gli3-/-. Gli3 is a regulator gene in the Hh pathway, mainly exerting repressive actions (Ruppert et al., 1988). Mutant mice lacking the Gli3 allele were first discovered at the Jackson's Lab, and these Gli3 animals can be used to model an upregulation of Shh pathway, as in some tissues Shh and Gli3 have opposing effects (Wang et al., 2000). It is the gene responsible for Greig

cephalopolysyndacryly syndrome (GCPS), Pallister-Hall syndrome (PHS) and Postaxial polydactyly type A (PAP-A) (Wild et al., 1997). Gli3 can be processed to act as an activator of transcription (Gli3A) in the presence of the Hh pathway activation, or as a transcriptional repressor in the absence of the Hh signalling. In many tissues, Gli3R acts to repress Shh transcription by repression of an intermediate transcriptional repressor (Weinreich and Hogquist, 2008).

4.2 Methods

Nine-week-old female Gli3+/- or Shh+/- dams were time-mated with a male Gli3+/- stud or Shh+/- stud respectively overnight, and the vaginal plugs were checked the following morning. The formation of a plug is counted as Embryonic Day (E) 0.5. Mice not plugged or pregnant were allowed to enter into the time-mating protocol again. At E17.5, the pregnant Gli3+/- mice were killed and embryos harvested. For the pregnant Shh+/- mice, they were dissected at E14.5. The foetuses were dissected under the microscope and analysed by flowcytometry using Accuri C6. The results from the embryos were litter mate controlled by normalisation against the mean measurements from WT embryo in the litter to eliminate inter-litter variations.

The results were analysed using Microsoft Excel, Prism and SPSS 22, and two-way ANOVA with post-hoc Tukey's multiple comparisons test was used as the chosen statistical test to allow for multi-comparisons.

4.3 Results

4.3.1. At E17.5, Gli3-/- embryos weigh more than the WT embryos, yet they have smaller livers.

At E17.5, the body mass of Gli3+/- embryos showed no statistical difference from the WT embryos, yet the Gli3-/- embryos had higher body mass compared to the WT embryos **(Figure 18A)**. When taken together, the bar charts appear to be a slight upward trend from WT embryos to Gli3+/-, and to Gli3-/-. The mean placenta mass for the three different genotypes of foetuses was not statistically different, and the FW:PW ratios were not different **(Figure 18B and C)**. However, there was greater variation in both placental mass and in FW:PW ratio in Gli3KO embryos compared to WT **(Figure 19B and C)**. Interestingly, even though Gli3-/- embryos were heavier, these foetuses had smaller livers, as the number of cells in the Gli3-/- liver was significantly lower than that in the WT livers **(Figure 18D)**. Likewise, the livers of Gli3+/- foetuses contained fewer cells than WT **(Figure 19D)**.

4.3.2. The immune profile of the livers from WT, Gli3+/- and Gli3-/- foetuses.

For the immune profile of the foetuses, the macrophage population (F4/80+ or CD11B+) did not show statistically significant differences among the three genotypes: WT, Gli3+/- and Gli3-/- **(Figure 19)**. Furthermore, this study did

not show a significant difference among the three groups for the CD3-NK1.1+ cell population **(Figure 20 A and B)**. Interestingly, for the CD3+NK1.1- population, which are T-cells, the results showed an increase in cell percentage in Gli3+/- foetal livers compared to the WT foetal livers. However, there was no statistical difference between the foetal livers from Gli3-/- embryos compared to the WT control **(Figure 20 C)**.

For the granulocytes, the percentage of GR1+LY6G+ cells was higher in the Gli3+/- and Gli3-/- foetal livers compared to WT **(Figure 21 A and B)**. In the case of the GR1+LY6G- population, although the mean percentage of GR1+LY6G- cells was greater in the Gli3 mutants, this difference was not statistically significant by student's t-test **(Figure 21 C)**.

For the B cell population, there were no differences in the B220+CD19- cell population among the three experimental groups **(Figure 22 A and B)**. However, for CD19+ cell population, the Gli3+/- foetal livers had a decrease in cell percentage compared to the WT; and there was a difference between Gli3-/- foetal livers and the control WT **(Figure 22C)**. The results are consistent with findings observed by Solanki et al, that deficiency in Gli3 reduced the percentage of B cells, and Shh negatively regulates B cell development, as an Shh mutation increased CD19+ B cells (Solanki et al., 2017)

4.3.3. At E14.5, Shh-/- had fewer liver cells than the WT embryos

We next investigated directly the impact of Shh mutation on immune cell development in the foetal liver, by timed mating Shh+/- adults. At E14.5, Shh-/- foetuses weighed less than the WT embryos or Shh+/- foetuses **(Figure 23A)**. However, Shh+/- foetuses showed no statistical differences compared with the WT foetuses **(Figure 23A)**. A similar trend was mirrored by the placental mass from the three different genotypes: the placentas from Shh-/- foetuses were smaller in size compared to the WT and Shh+/- foetuses, and WT and Shh+/- placentas were similar **(Figure 23B)**. For FW:PW ratio, Shh-/- embryos had a lower ratio compared to WT embryos and Shh+/- embryos **(Figure 24 C)**. Shh-/- livers contained fewer cells than WT or Shh+/- **(Figure 24D)**.

4.3.4 The foetal liver innate immune profile of WT, Shh+/- and Shh-/- embryos at E14.5.

At E14.5, the macrophage population was identified by CD11B and F4/80 staining. There were no significant differences among the three genotype groups **(Figure 24A)**. When the cells were analysed for surface GR1 expression, a deficiency in shh led to decreased granulocyte population **(Figure 25A & B).**

There was also no detectable difference for the NK1.1+ population for the three experimental groups **(Figure 26 A & B)**. For the CD3+NK1.1- cell population, which marks the T-cells, foetal livers from Shh-/- embryos had significantly lower percentage of CD3+ cells compared to both the WT control and Shh+/- embryos **(Figure 26 A & C)**.

A. Foetal weight at E17.5

	Mean difference	p-value
WT vs Gli3+/-	-0.049	0.26
WT vs Gli3-/-	-0.154	<0.05*
Gli3+/- vs Gli3-/-	-0.105	0.183

B. Placental weight at E17.5

	Mean difference	p-value
WT vs Gli3+/-	-0.0892	0.741
WT vs Gli3-/-	0.0375	0.958
Gli3+/- vs Gli3-/-	0.127	0.661

C. Foetus to placenta ratio

	Mean difference	p-value
WT vs Gli3+/-	1.17	0.326
WT vs Gli3-/-	-0.145	0.984
Gli+/- vs Gli3-/-	-1.315	0.364

D. Foetal liver cell number

	Mean difference	p-value
WT vs Gli3+/-	18493200	0.463
WT vs Gli3-/-	49904400	0.005
Gli3+/- vs Gli3-/-	31411200	0.03

Figure 18: Characteristics of WT, Gli3+/- and Gli3-/- foetuses born to Gli3+/- mothers on E17.5.

(A) Bar chart showing the mean relative weight of the foetuses with different genotypes; (B) Bar chart showing the mean relative placental mass of the foetuses with different genotypes; (C) Bar chart showing mean FW:PW ratio of the foetuses of different genotypes; (D) Bar chart showing the mean number of liver cells for each genotype. *$p < 0.05$, n=13, error bar±SEM.

A. Macrophage population

B. Macrophage percentage

	Mean difference	p-value
WT vs Gli3+/-	0.0521	0.964
WT vs Gli3-/-	-0.0758	0.937
Gli3+/- vs Gli3-/-	-0.128	0.824
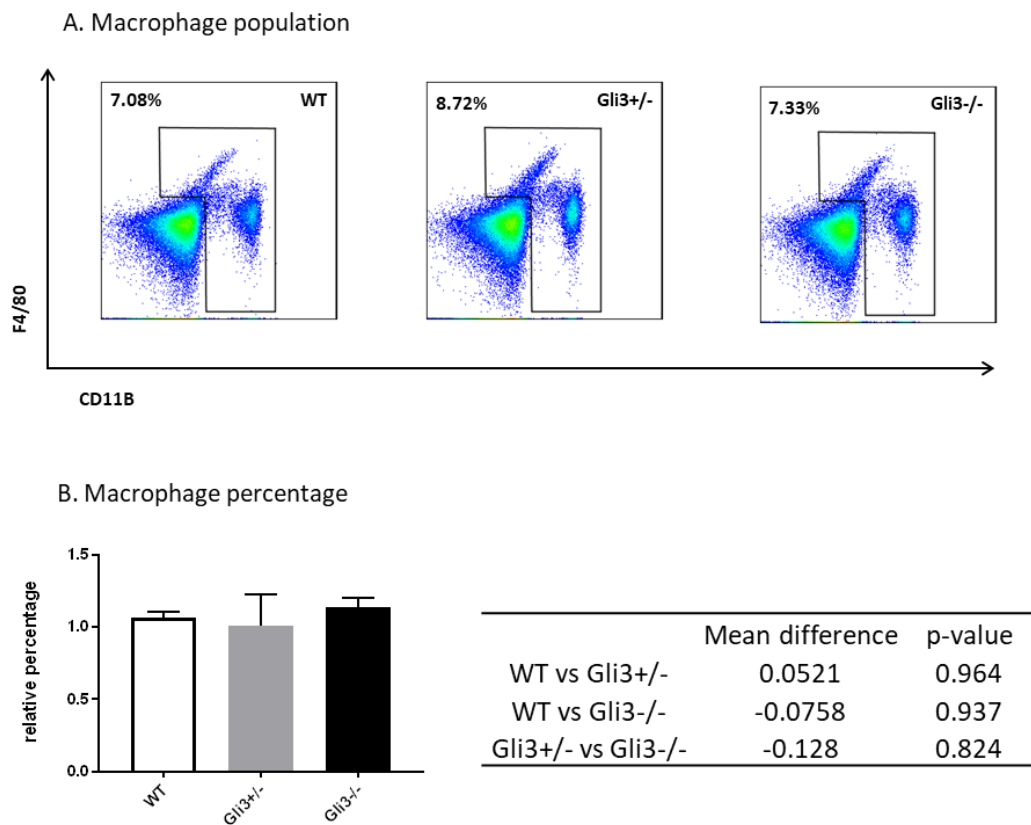
Figure 19: The macrophage population marked by F4/80+ or CD11B+ in foetal livers of the three genotypes on E17.5.

(A) Flow cytometric diagram showing the mean percentage of macrophages in foetal livers with different genotypes; (B) bar chart showing the macrophage population in the foetal livers with different genotypes. n=11, error bar±SEM.

A. CD3+NK1.1- and CD3-NK1.1+ cells

B. CD3-NK1.1+ cell percentage

	Mean difference	p-value
WT vs Gli3+/-	0.0917	0.932
WT vs Gli3-/-	-0.257	0.629
Gli3+/- vs Gli3-/-	-0.342	0.36

C. CD3+NK1.1- cell percentage

	Mean difference	p-value
WT vs Gli3+/-	-1.23	0.007*
WT vs Gli3-/-	-0.579	0.129
Gli3+/- vs Gli3-/-	0.656	0.112
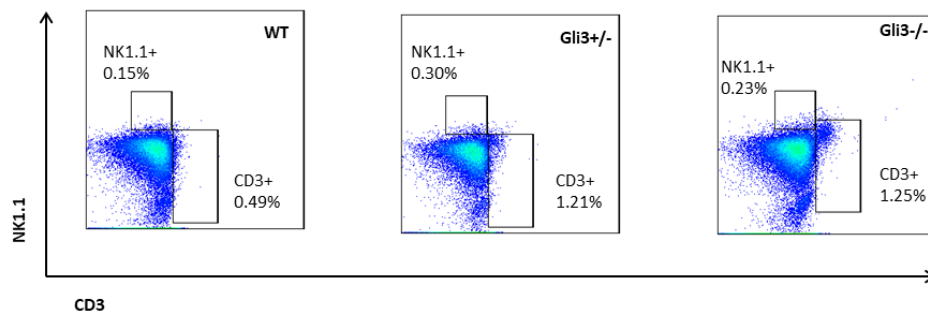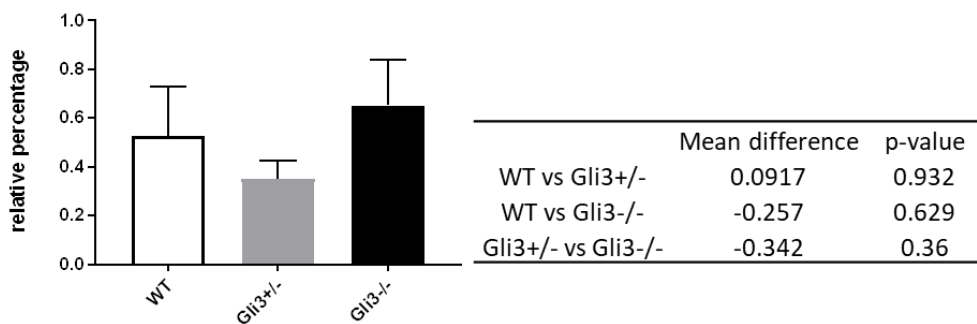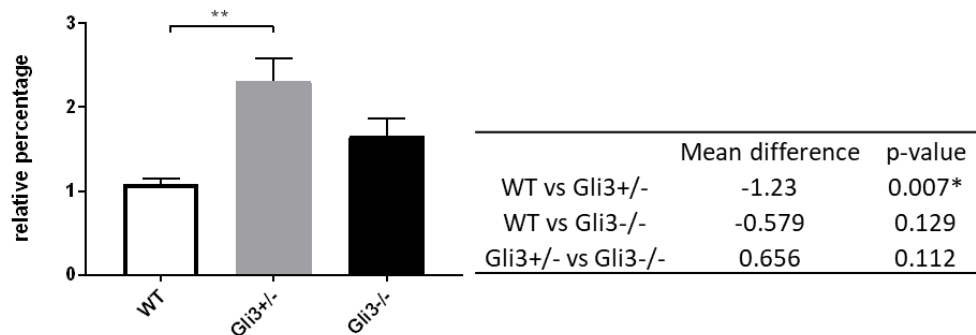
Figure 20: NK cell population in offspring born to Gli3+/- mothers at E17.5:

(A) flow cytometry of NK cell populations in WT, Gli3+/- and Gli3-/- foetuses; (B) bar chart showing mean relative percentage of NK1.1+ cell population from the three groups; (C) Bar chart showing CD3+ mean percentage from the three groups. **p<0.01, n=13, error bar±SEM.

A. Neutrophil and eosinophil cell population

B. Neutrophil cell percentage

	Mean difference	p-value
WT vs Gli3+/-	-0.462	0.0142*
WT vs Gli3-/-	-0.56	0.0155*
Gli3+/- vs Gli3-/-	-0.0977	0.813

C. Eosinophil cell percentage

	Mean difference	p-value
WT vs Gli3+/-	-0.249	0.0673
WT vs Gli3-/-	-0.283	0.0641
Gli3+/- vs Gli3-/-	-0.0337	0.946
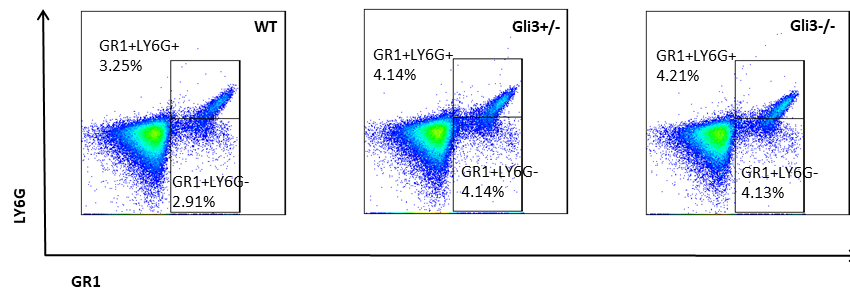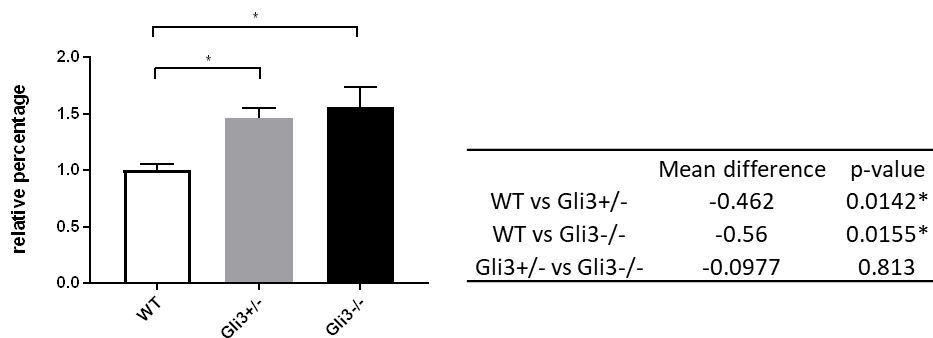
Figure 21: Percentage of GR1+LY6G+ cell population and GR1+LY6G-

cell populations in foetuses of Gli3+/- mothers at E17.5.

(A) flow cytometry showing GR1+LY6G+ and GR1+LY6G- cell populations of

the foetuses with different genotypes; (B) bar chart showing GR1+LY6G+ cell

population of the foetuses with different genotypes; (C) bar chart showing

GR1+LY6G- cell population of the foetuses with different genotypes. *p<0.05,

n=15.

A. CD19 and B220 cell population

WT	Gli3+/-	Gli3-/-
B220+ 0.62%	B220+ 0.74%	B220+ 0.99%
CD19+ 5.99%	CD19+ 7.10%	CD19+ 6.85%

B. CD19+ cell percentage

	Mean difference	p-value
WT vs Gli3+/-	0.15	0.395
WT vs Gli3-/-	-0.0123	0.994
Gli3+/- vs Gli3-/-	-0.163	0.228

C. B220+ cell percentage

	Mean difference	p-value
WT vs Gli3+/-	0.181	0.0046
WT vs Gli3-/-	0.124	0.0944
Gli3+/- vs Gli3-/-	-0.0569	0.472
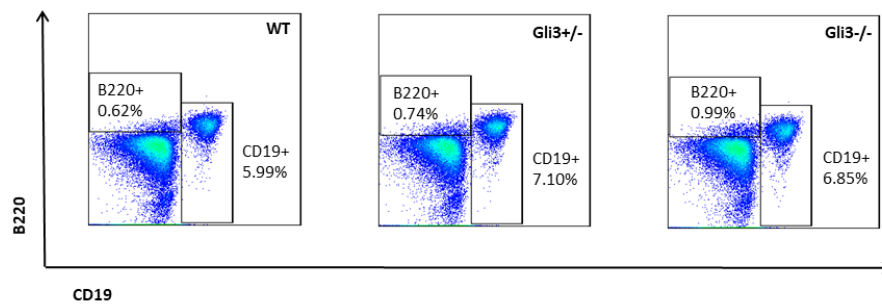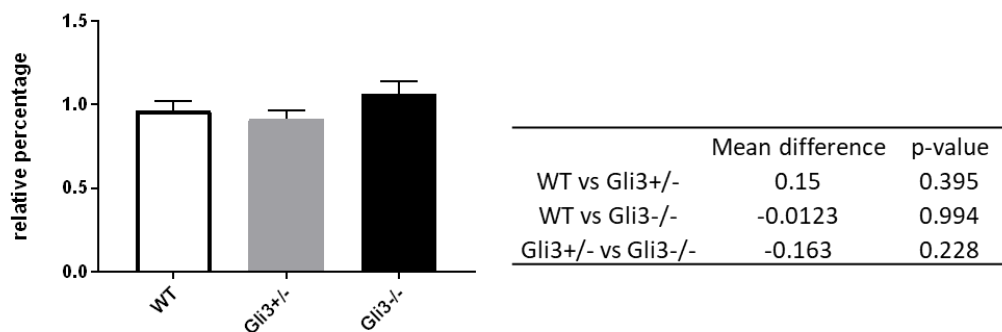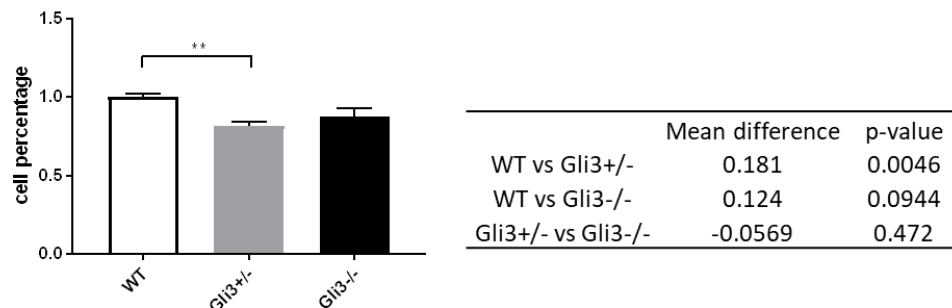
Figure 22: B cell population in WT, Gli3+/- and Gli3-/- foetuses at E17.5.

(A) flow cytometric diagram showing B220+ and CD19+ populations of the foetuses with different genotypes; (B) Bar chart showing the mean percentage of B220+CD19- populations of the foetuses with different genotypes; (C) Bar chart showing the mean percentage of CD19+ cells in foetuses with different genotypes. *p<0.05, **p<0.005 n=14.

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A. Foetal weight at E14.5

	Mean difference	p-value
WT vs Shh+/-	0.019	0.891
WT vs Shh-/-	0.624	<0.01**
Shh+/- vs Shh-/-	0.605	<0.01**

B. Placental weight at E14.5

	Mean difference	p-value
WT vs Shh+/-	-0.00875	0.998
WT vs Shh-/-	0.457	<0.05*
Shh+/- vs Shh-/-	0.465	<0.05*

C. Foetus to placenta ratio

	Mean difference	p-value
WT vs Shh+/-	0.462	0.291
WT vs Shh-/-	1.082	<0.05*
Shh+/- vs Shh-/-	0.62	0.159

D. Foetal liver cell number

	Mean difference	p-value
WT vs Shh+/-	3957800	0.463
WT vs Shh-/-	5060069	0.005**
Shh+/- vs Shh-/-	11102269	0.03*

Figure 23: The characteristics of foetuses from Shh+/- mothers at E14.5.

(A) bar chart showing the body mass of WT, Shh+/- and Shh-/- foetuses at E14.5 (B) bar chart showing the placental mass of the three genotype groups at E14.5; (C) bar chart showing the FW:PW ratio of the three genotype groups at E14.5; (D) bar chart showing the whole liver cell numbers of the three genotype groups at E14.5. *p<0.05, **p<0.01, error bar ±SEM.

A. Macrophage population

B. Macrophage percentage

	Mean difference	p-value
WT vs Shh+/-	0.27	0.102
WT vs Shh-/-	0.0581	0.937
Shh+/- vs Shh-/-	-0.212	0.413
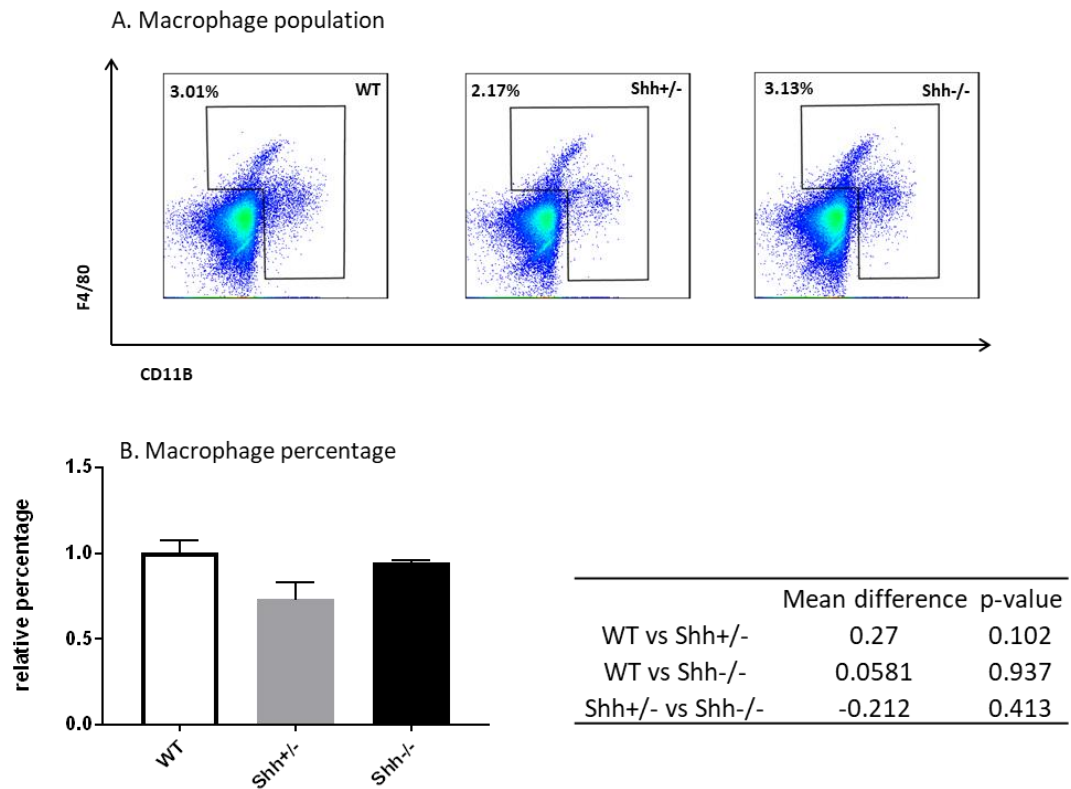
Figure 24: The immune profile of foetuses from Shh+/- mothers at E14.5.

(A) flow cytometric diagram showing the macrophage population; (B) bar chart showing the macrophage cell percentage among the three groups. n=13, error bar ±SEM.

A. Granulocyte population

B. Granulocyte percentage

	Mean difference	p-value
WT vs Shh+/-	0.277	0.0223*
WT vs Shh-/-	0.235	0.155
Shh+/- vs Shh-/-	-0.0417	0.929
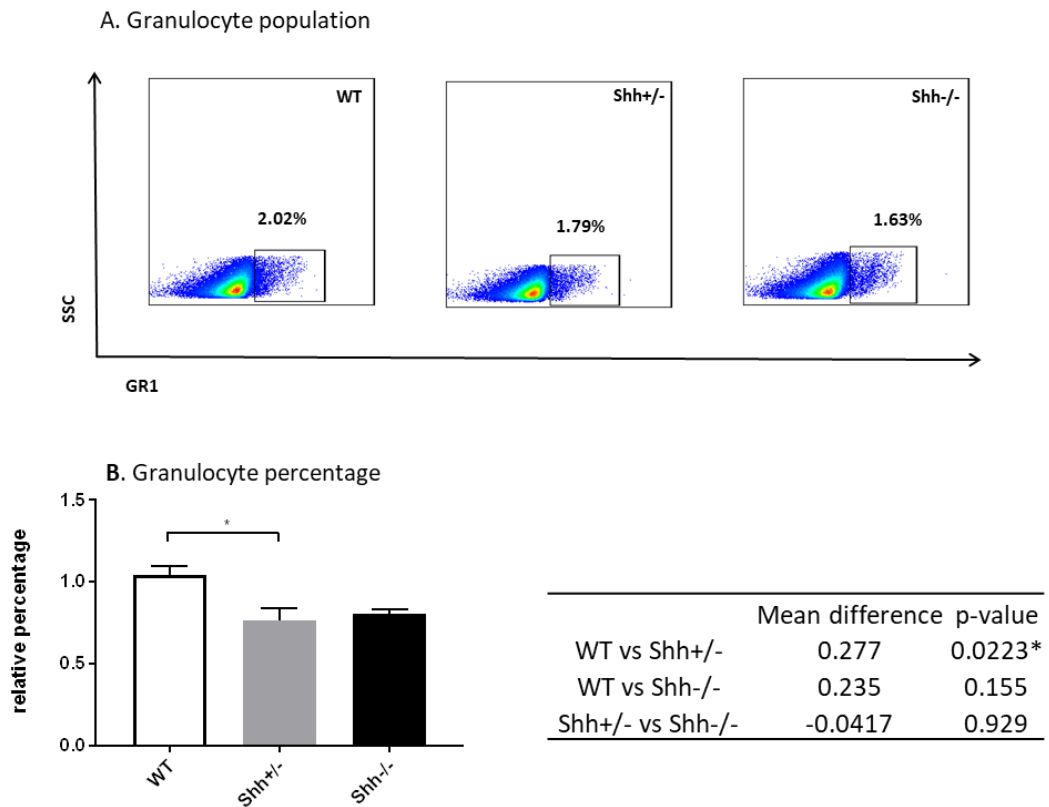
Figure 25: Granulocyte population in WT, Shh+/- and shh-/- foetuses at E14.5.

(A) flow cytometric diagram showing granulocyte population among the three genotypes; (C) bar chart showing the granulocyte cell percentage in the three groups. n=11, error bar ±SEM.

A. CD3+NK1.1- and CD3-NK1.1+ cells

B. CD3-NK1.1+ cell percentage

	Mean difference	p-value
WT vs Shh+/-	0.322	0.17
WT vs Shh-/-	-0.155	0.834
Shh+/- vs Shh-/-	-0.472	0.195

C. CD3+NK1.1- cell percentage

	Mean difference	p-value
WT vs Shh+/-	-0.08	0.926
WT vs Shh-/-	0.893	0.0139*
Shh+/- vs Shh-/-	0.973	0.046*
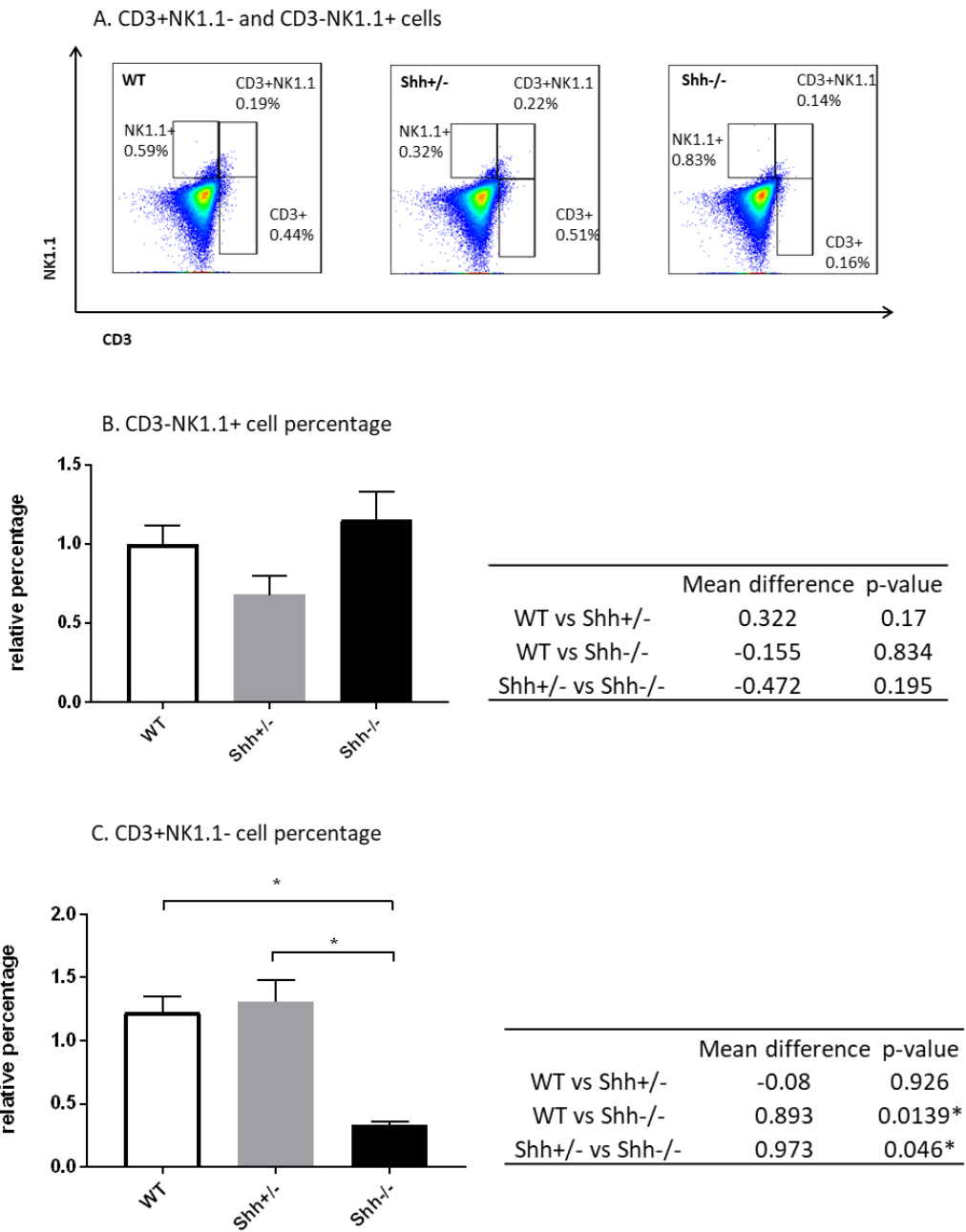
Figure 26: NK cell population in WT, Shh+/- and shh-/- foetuses at E14.5.

(A) flow cytometric diagram showing CD3+, and/or NK1.1+ cell population; (B) bar chart showing the NK1.1+ cell population among the three different groups; (C) bar chart showing CD3+ cells in the three groups. *p<0.05, n=13, error bar ±SEM.

4.4 Discussion

This experiment serves as a continuation of the findings in **Chapter 3,** where the foetuses from obese mothers had elevated *Gli3* gene expression. To further investigate the general hypothesis that the immune system, Hh and maternal dietary status interact with each other, it was first necessary to establish if Hh plays a role in the development of the innate system in foetal livers. The results suggest that Shh and Gli3 have a role in the control of the size of the foetuses, since mutation of Gli3 in the case of Gli3+/- and Gli3-/- embryos, increased the size of the foetus, but decreased number of foetal liver cells. The lack of the *Gli3* gene also led to an increase in the granulocyte population, increase in neutrophil population and a decrease in B220+ cell population, whereas the mutation of *Shh* led to reduced body mass, decreased granulocyte population and decreased CD3+ population. The observations for granulocytes suggest the consistency that Gli3 and Shh have opposite effects on development and further confirmed the important function of Hh as a morphogen in determining foetal development.

In this experiment, the innate immune cells such as macrophage, NK cells, neutrophils and eosinophils were examined, which are the cells implicated in the pathogenesis of NAFLD (Zhan and An, 2010). Only the neutrophils appeared to be affected by the different genetic background. The other cell group which was shown to have a significant difference were the lymphocytes. Both B cells and T cells were affected.

The evidence that Hh is involved in the development of embryonic organs are well-described, yet there is conflicting evidence on the extent Hh exerts an effect on haematopoiesis. Namely, Gao et al has shown that alteration of Hh pathway by conditional Smoothened (Smo) deletion or overactivation did not affect HSCs self-renewal and function, suggesting that Hh signalling pathway is dispensable (Gao et al., 2009). Hofmann et al also showed that conditional deletion of Smo did not affect repopulation of HSCs (Hofmann et al., 2009). Yet other studies have suggested a role of Hh in the development of haematopoietic stem cells, with evidence from zebra fish that mutation of Hh led altered HSCs formation (Gering and Patient, 2005). Also, several studies have linked Hh pathway in orchestrating the development of T cells and B cells, which are all from the lymphoid lineage, or the adaptive immune system (Solanki et al., 2017, Furmanski et al., 2012, Saldana et al., 2016, Shah et al., 2004). Yet, only limited number of studies have suggested the role of Hh in the fate of the innate immune cells However, it is also important to note, that HSCs in adults may behave differently than from the foetuses, hence the conflicting results. Alternatively, Hh signalling in haematopoietic cells may be Smo-independent.

The predominant role of Gli3 is to repress the Hh Signalling Pathway but Gli3 can act as a transcriptional activator. Additionally, Gli3 can function both dependently and independently of Hh. Therefore, it was important to test the

hypothesis by using both Gli3 and Shh mutant mice, as Shh deficient mice and Gli3 deficient mice are known to have opposing effects (Wang et al., 2000). However, further work is required to delineate the exact mechanisms involved in the findings of this chapter.

4.5 Conclusion

In this study, the experiments did not show Hh Signalling pathway having major effects on macrophages and NK cells, which are implicated in the pathogenesis of NAFLD. However, Hh signalling pathway appeared to influence the GR1+LY6+ cell population, B cells and T cells.

Chapter 5: The Hedgehog signalling pathway and Non-alcoholic Fatty Liver Disease

5.1. Introduction

5.1.2. Role of the hedgehog signalling pathway in adult NAFLD.

The Hedgehog (Hh) signalling pathway is an important pathway involved in organogenesis and morphogenesis. It is highly conserved across species and is most active during early development stage. The number of Hh producing cells and Hh responsive cells also decrease postnatally (Petrova and Joyner, 2014). In adults, it is reactivated, when there is an injury (Omenetti et al., 2011). In the liver, the following cells produce Hh proteins: hepatocytes, cholangiocytes, hepatic stellate cells, natural killer T cells and sinusoidal endothelial cells; with hepatocytes a major source of Hh in NAFLD. Many studies have addressed the involvement of Hh in adult NAFLD, with research focusing on the fibrosis stage of NAFLD: NASH. A study by Swidersky-Syn et al has demonstrated that Shh immunohistochemistry expression is correlated with the fibrosis stage (Swiderska-Syn et al., 2013). Similarly, rodent models of NAFLD were shown to exhibit increased Hh-ligand concentration, and increased expression of the Gli-target gene *Osteopontin* (Syn et al., 2011). In a post hoc evaluation of the Pioglitazone versus Vitamin E versus Placebo for the Treatment of Nondiabetic Patients with Nonalcoholic Steatohepatitis (PIVENS) trial, loss of Shh expressing hepatocytes strongly correlated with treatment response (Guy et al., 2015). It has been shown that liver-specific

inhibition of Smo, protected against liver injury and liver fibrosis in different dietary models of NASH (Verdelho Machado and Diehl, 2016).

In *in vitro* studies, cultured mouse NKT (DN32 iNKT) cells were shown to produce and respond to Shh, and co-culture with recombinant Shh enhanced proliferation, decreased apoptosis in this NKT cells line and increased expression of profibrogenic cytokine IL-13 (Syn et al., 2009). Similarly, several studies have drawn a link between Hh and obesity, which is the major risk factor leading to NAFLD (Teperino et al., 2014, Pospisilik et al., 2010a). Additionally, it has been shown that Hh pathway blocks early steps of adipogenesis and alters adipocyte morphology and insulin sensitivity (Cousin et al., 2007).

5.1.3. The possible mechanism linking Hh, NAFLD and inflammatory immune cells.

In adults, when there is injury or damage to the liver, the Hh pathway is activated and immune cells are also recruited. However, depending on the level and duration of the injury, the inflammatory response may be the cause of further injuries or may participate in repairs. There is evidence that chronic inflammation, such as obesity, leads to aberrant extracellular matrix modelling (Arngrim et al., 2013). The immune response may have pivotal roles in fibrogenesis in the liver. During the process, both classical immune cells, such

as neutrophils, macrophages and NK cells; and non-classical immune cells such as hepatocytes, hepatic stellate cells, endothelial cells are involved (Arrese et al., 2016). Hh plays a role in the phenotype and function of the immune cells. It has been shown that Hh directly induces M2 pro-fibrogenic polarisation of macrophages/Kupffer cells.

Aim: the aim of this chapter is investigating the involvement of Hh in adult NAFLD

5.2. Method

In this study, there are three experimental groups of adult male mice: wild-type (WT), Gli3+/- and WT mice injected with Smo inhibitors. All mice were fed an obesogenic diet for 20 weeks. The weights were recorded weekly. At the end of 20 weeks, the mice were fasted overnight, then given a bolus of 20% glucose at 1ml per gram of the initial body weight of the animal. The blood glucose was tested with a glucometer at the following time-points: 0 min, 15 mins, 30 mins, 60 mins, 90mins and 120 mins. Liver, perigonadal fat and inter-scapular fat were then collected and snap frozen for later analyses. The blood was collected from the inferior vena cava, centrifuged at 4 °C for 15 mins at 12000 rcf. Some of the WT mice given an injection of the Smo inhibitor 40µ per day in DMSO (control is vehicle only) for 6 weeks after 20 weeks on HFD.

The livers of these mice were digested with collagenase and DNase, following the adult liver protocol outlined in the **Chapter Two General Materials and Methods**. The cells were then stained for the following markers and analysed by LSRII. As Ly-6C is rare in terms of cell population, FMO control for PCP-cy5.5 Ly6C was used in this instance.

Colour	Marker
APC	F4/80
PCP-cy5.5	Ly-6C
Alexa 488/FITC	CD11B
PE	CD11C
PE/Cy5	CD3
Brilliant violet 650	CD19
Pacific Blue	Ly6G
Alexa Fluor 700	CD45
PE-Cy7	Nk1.1
Am Cyan	Live/Dead

Table 5. Stain panel for flow cytometry

5.3. Results

5.3.1. Characteristics, weight, liver weight and glucose tolerance of WT and Gli3+/- male mice fed on a high-fat, high-sugar diet.

The weight change from the baseline weight, which is the weight of the mice before entering into the feeding protocol, was recorded weekly for the both groups and it was observed that at the beginning of the feeding, the weight change was similar in both groups. Yet, over the span of 20 weeks, WT mice appeared to gain weight more rapidly than the Gli3+/- mice. However, towards the end of the 20 weeks, the weight change in the WT groups started to plateau, showing that the final weight reached by both WT and Gli3+/- groups was similar **(Figure 27 A)**.

Immediately before dissection, all the mice were subjected to an oral glucose tolerance test, and the Gli3+/- group had a higher peak at 15 minutes compared to the WT **(Figure 27 B)**, and the area under the curve (AUC) was also higher in the Gli3+/- group compared to the WT group **(Figure 27 C).**

For the ratio between liver mass and body mass, expressed in percentage (%), Gli3+/- has a lower percentage compared to the WT group **(Figure 27 D)**. Moreover, there appeared to be no statistical difference between the WT and Gli3+/- group for mass of the white adipose tissue (WAT).

5.3.2. The immune profile of WT and Gli3+/- male mice fed on a high-fat, high-sugar diet.

Flow cytometric analyses of the liver showed that Gli3+/- mice had increased granulocytes (LY6G+) cell population compared to the WT controls **(Figure 28 A and B)**. However, other cell populations were unaffected by the different genetic backgrounds. There was no difference in the macrophage population in the groups **(Figure 28 C and D)**. The LY6C+ macrophage population was not significantly different **(Figure 28 E and F)**. For the NK cell population, both WT and Gli3+/- groups showed similar cell percentages **(Figure 28 G and H)**.

5.3.3. Characteristics, weight, liver weight and glucose tolerance of WT male mice fed a high-fat, high-sugar diet, with or without Smo inhibitor treatment.

At the end-point, obese mice treated with Smo inihibitor and obese mice without the Smo inhibitor treatment, exhibit similar characteristics. Body mass, liver to body mass ratio, glucose tolerance curve, peak glucose reading, area under the curve and the WAT mass all did not exhibit statistical significance. This implies the obese mice which underwent Smo inhibitor treatment did not show any change phenotypically **(Figure 29)**.

However, the immune profile of livers from these two groups was affected by the Smo inhibitor treatment. Firstly, the treated group appeared to have a reduced LY6G+ population **(Figure 30 A and B)**. The monocyte population also was affected: there was a reduction in the percentage of CD11B+ cells in the treated group compared to the non-treated group **(Figure 30 C and D)**. For the overall macrophage population, the percentage of cells did not show a difference between the two experiment groups. Yet, out of thesis macrophage population, the LY6C+ macrophage population was reduced by the Smo inhibitor treatment **(Figure 31 G and H)**.

For the CD3-NK1.1+ population, there appeared to be no difference between the treated and untreated groups **(Figure 31 A and B)**. Yet, interestingly, the treated group had an increase in B cell population compared to the untreated group **(Figure 32 C and D)**.
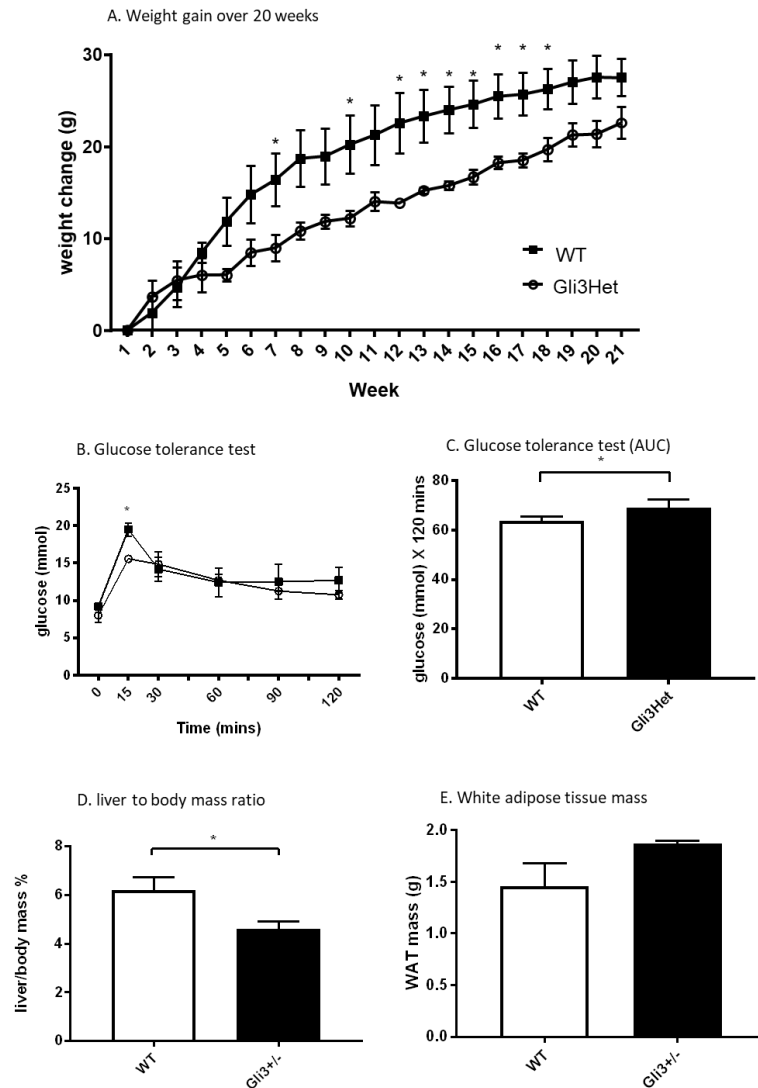
Figure 27: characteristics of WT and Gli3+/- male adult mice fed on HFD.

(A) weekly weight change of WT and Gli3+/- male mice on HFD; (B) glucose tolerance test of WT and Gli3+/- male mice on HFD; (C) bar chart showing area under the under for the glucose tolerance test for both WT and Gli3+/- male mice on HFD; (D) liver to body mass ratio for both WT and Gli3+/- male mice on HFD; (E) White adipose tissue mass for WT and Gli3+/- male mice on HFD. *p<0.05, n=4.

A. Ly6G+ and Ly6G- cells

B. Ly6G+ cell percentage

C. Macrophage, gated on Ly6G-

D. Macrophage cell percentage, gated on Ly6G-

E. Ly6C+ Macrophage

F. Ly6C+ Macrophage cell percentage

G. NK1.1+ cells

G. NK1.1+ cell percentage

123
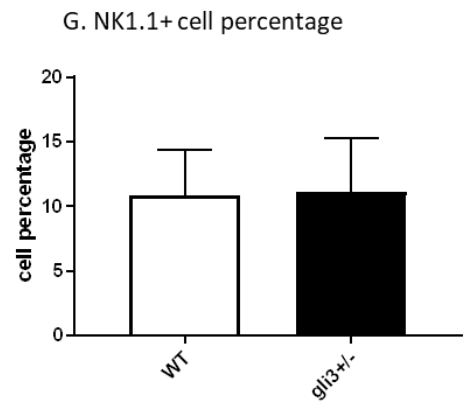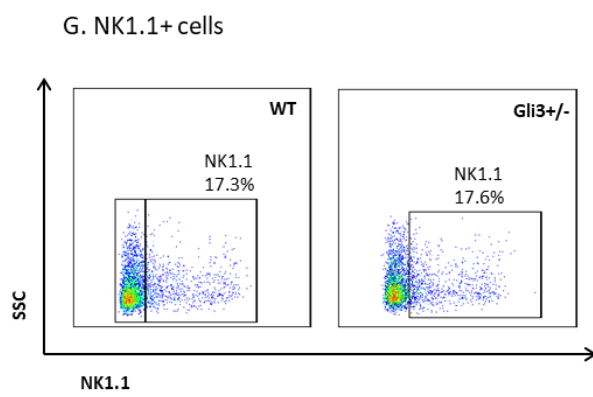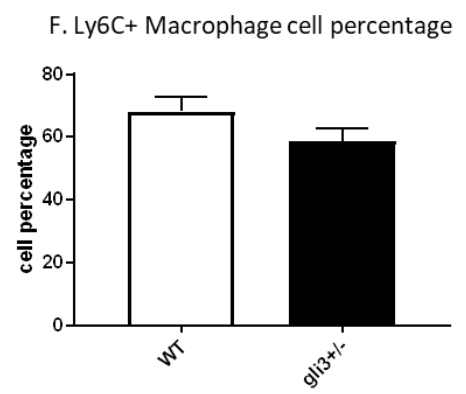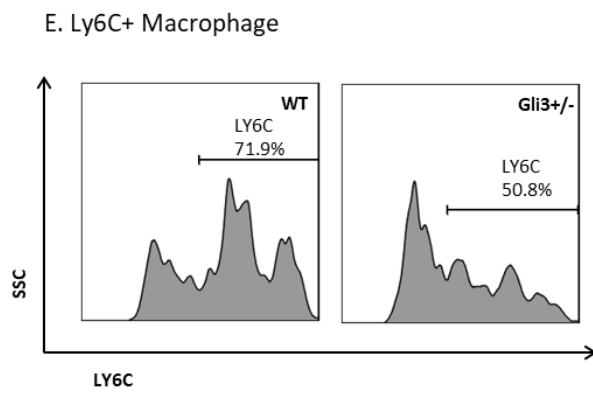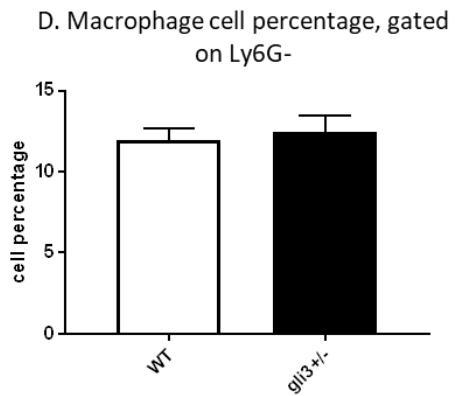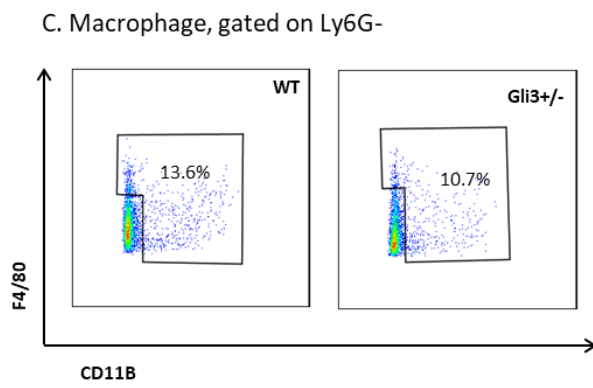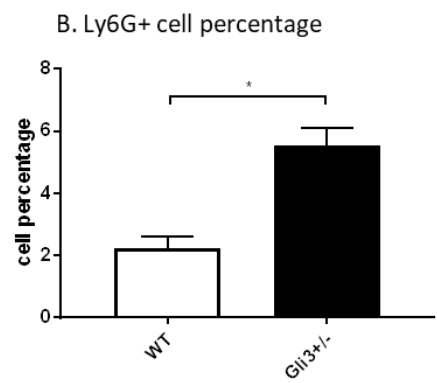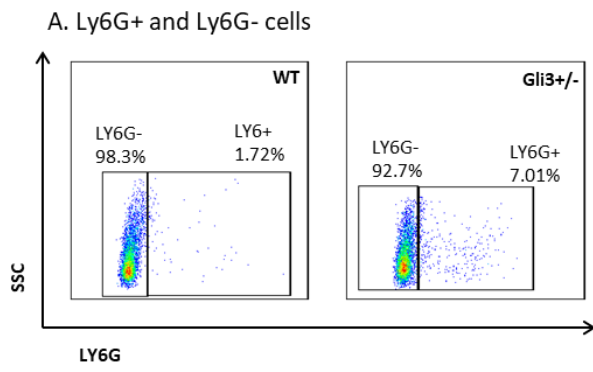
Figure 28: The immune profile of WT and Gli3+/- male mice fed on HFD.

(A) Flow cytometric diagram showing Ly6G- and Ly6G+ cell population from WT and Gli3+/- mice; (B) bar chart showing mean percentage of Ly6G+ population; (C) flow cytometric diagram showing mean percentage of macrophage population; (D) bar chart showing the mean percentage of macrophage population; (E) flow cytometry histogram showing LY6C+ population; (F) bar chart showing mean percentage of LY6C+ population in WT and Gli3+/- mice; (G) flow cytometric diagram showing NK1.1 cell population in WT and Gli3+/- mice; (H) bar chart showing NK1.1+ cell population from WT and Gli3+/- mice. *p<0.05, n=4

A. Body mass

B. Liver to body mass ratio

C. Glucose tolerance test

D. Glucose tolerance test (AUC)

E. Peak glucose

F. White adipose tissue mass
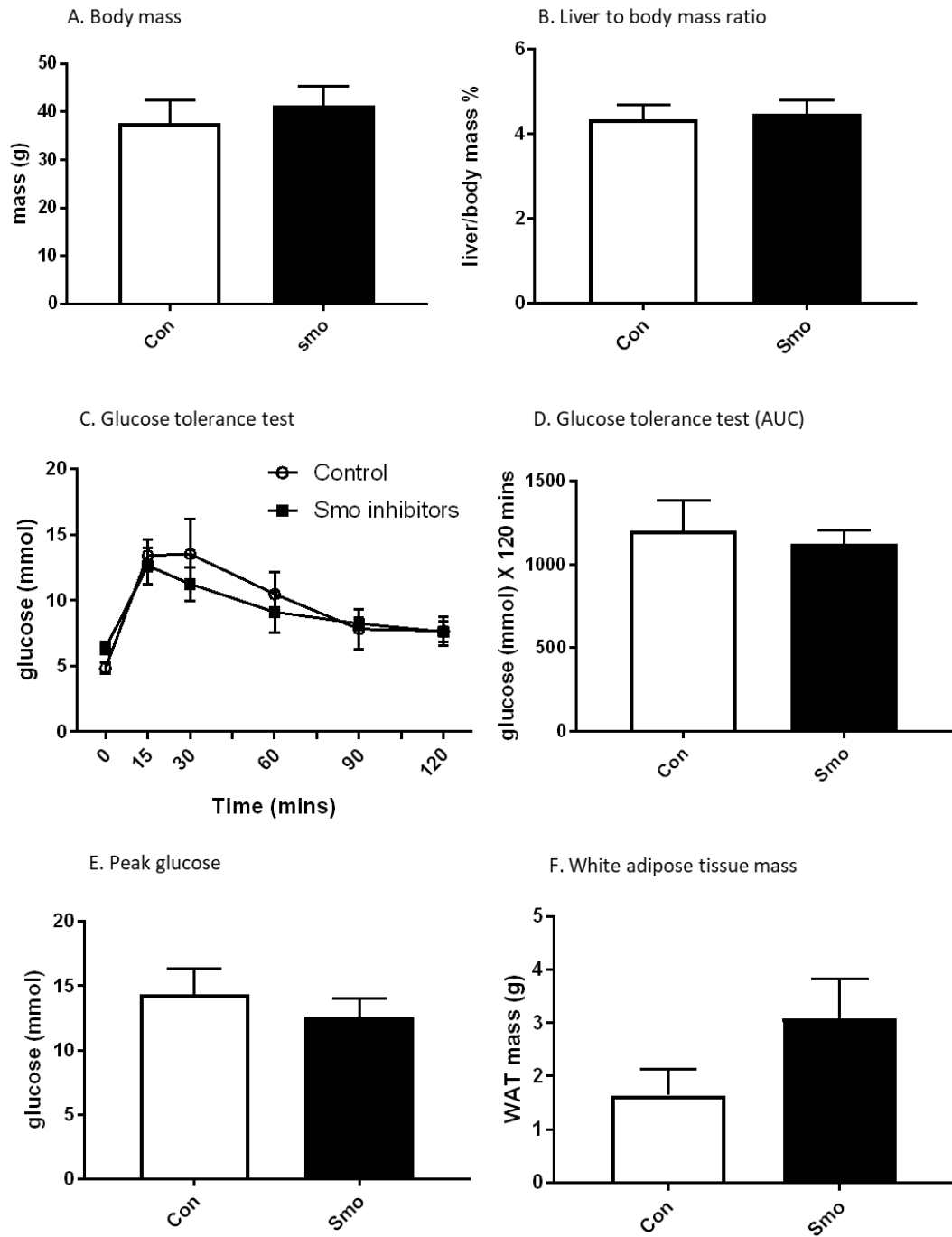
Figure 29: Characteristics of male mice fed HFD and treated with Smo inhibitors.

(A) bar chart showing the mean body mass of WT on HFD treated with Smo inhibitor and control WT; (B) bar chart showing liver/body mass ratio of WT on HFD treated with Smo inhibitor and control WT; (C) glucose tolerance test of WT fed on HFD treated with Smo inhibitor and control WT; (D) bar chart showing mean area under the curve of WT fed on HFD treated with Smo inhibitor and control WT; (E) bar chart showing mean peak glucose from WT fed on HFD treated with Smo inhibitor and control WT; (F) bar chart showing mean WAT mass from WT fed on HFD treated Smo inhibitor and control WT. n=4. Error bar±SEM.

A. Ly6G+ and Ly6G- cells
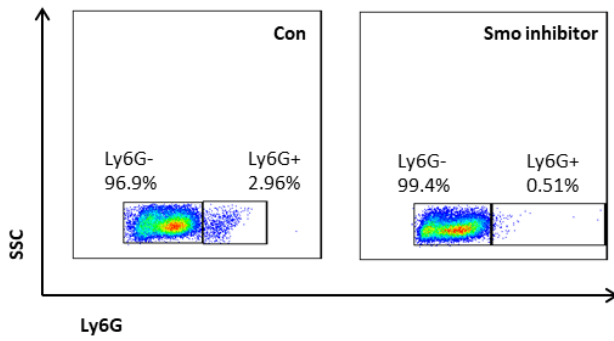
B. Ly6G+ cell percentage
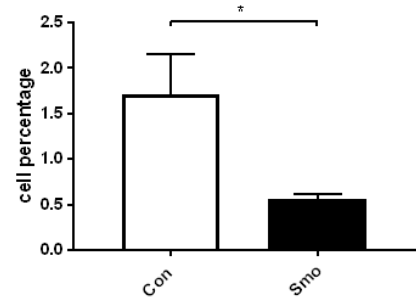
C. CD11B+, gated on Ly6G-
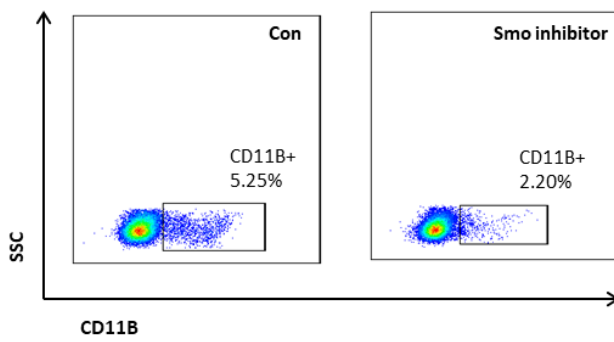
D. CD11B+ cell percentage, gated on Ly6G-
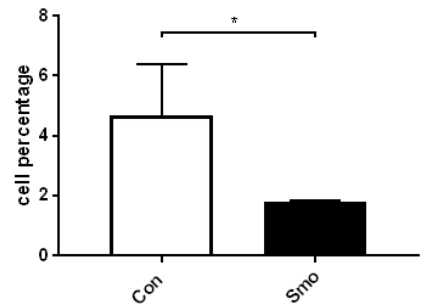
E. Macrophage, gated on Ly6G-
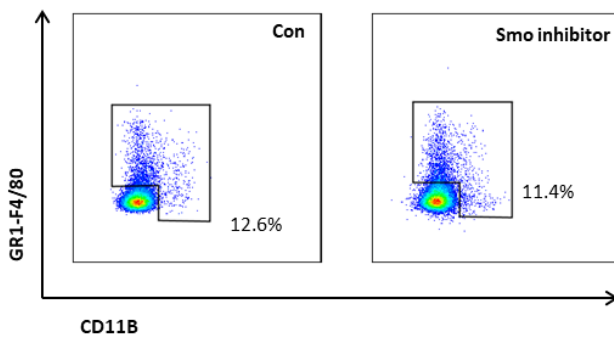
F. Macrophage cell percentage, gated on Ly6G-
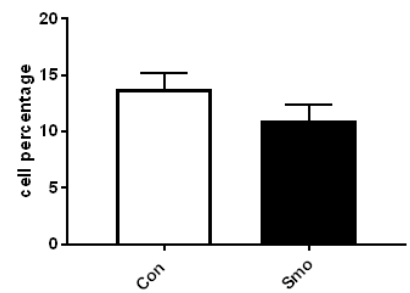
G. Ly6C+ Macrophage
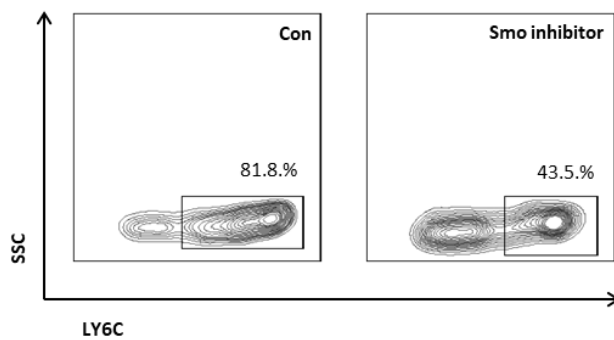
H. Ly6C+ macrophage cell percentage
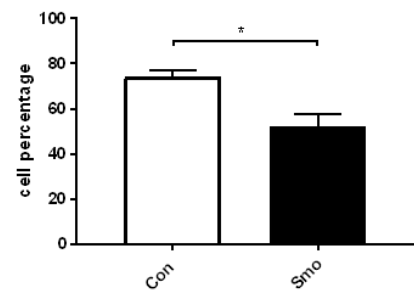
Figure 30: The immune profile of WT male mice fed on HFD vs WT male mice on HFD and treated with Smo inhibitor.

(A) flow cytometric diagram showing the Ly6G+ population in WT mice fed on HFD and WT mice on HFD treated with Smo inhibitor; (B) bar chart showing mean percentage of GR1+ population in mice on HFD treated with Smo inhibitor; (C) flow cytometric diagram showing mean percentage of CD11B+ cell population in WT on HFD and WT mice on HFD treated with Smo inhibitor; (D) bar chart showing CD11B+ cell population ion mice on HFD treated with smo inhibitor; (E) flow cytometric diagram showing macrophage population in WT mice on HFD and WT mice on HFD treated with Smo inhibitor; (F) bar chart showing mean percentage of F4/80+ and CD11B+ cells in WT mice fed on HFD and WT mice fed on HFD treated with Smo inhibitors; (G) flow cytometric diagram showing Ly6C+ cell population in WT mice on HFD and WT mice on HFD treated with Smo inhibitor; (H) bar chart showing mean percentage of Ly6C+ population in WT mice fed on HFD treated by Smo inhibitor. *$p < 0.05$, n=4, Error bar±SEM.
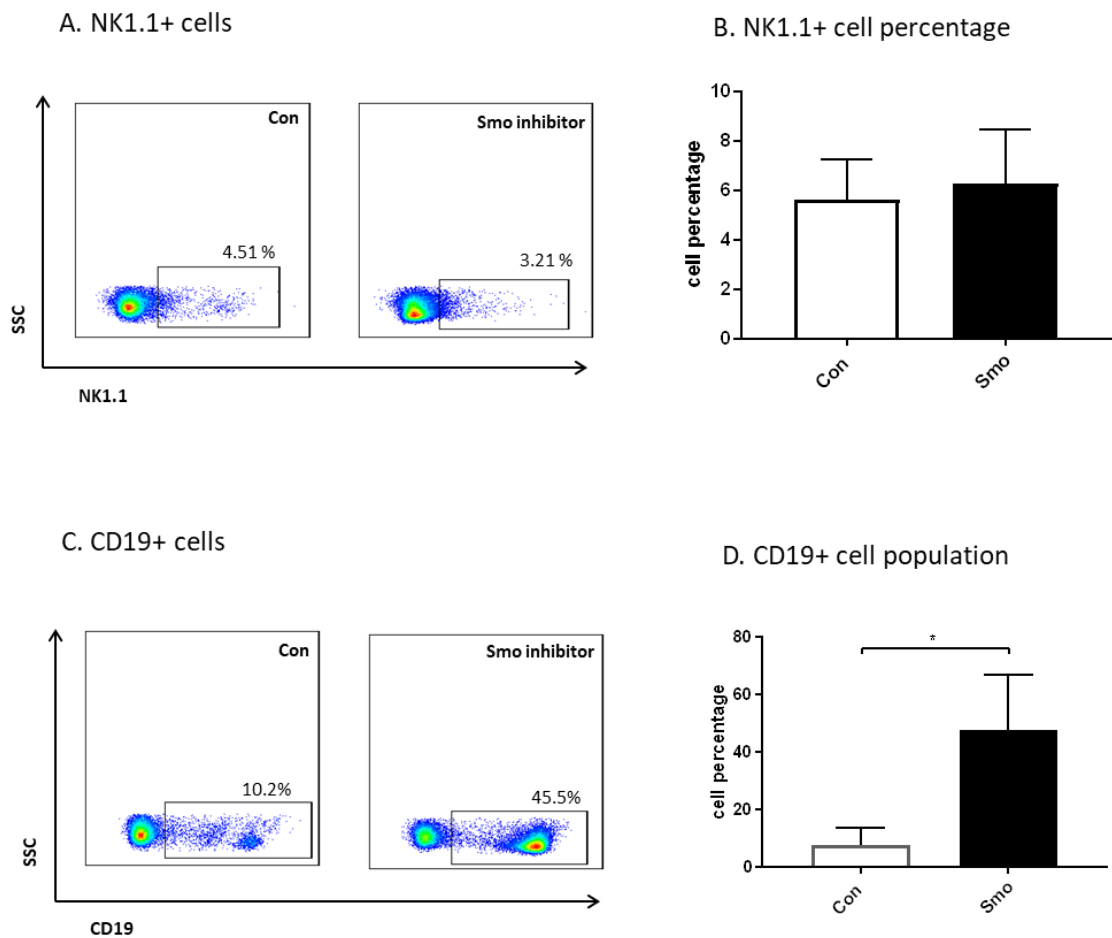
Figure 31: NK1.1+ and CD19+ cell population in the livers of WT male mice fed on HFD vs WT male mice fed on HFD treated with Smo inhibitor.

(A) flow cytometric diagram showing NK1.1+ cell population in WT mice fed on HFD, and WT mice fed on HFD treated with Smo inhibitor; (B) bar chart showing mean percentage of NK1.1+ cells in WT mice fed on HFD and WT mice fed on HFD treated with Smo inhibitor; (C) flow cytometric diagram showing CD19+ cell population in WT mice fed on HFD, and WT mice fed on HFD treated with Smo inhibitor; (D) bar chart showing mean percentage of CD19+ cell population in WT mice fed on HFD treated with Smo inhibitor. *$p < 0.05$, n=4, Error bar±SEM.

5.4. Discussion

Macrophages and NK cells have been implicated in the pathogenesis of NAFLD. In this study, the immune profiles of the livers from obese WT, obese Gli3+/- and obese WT treated with Smo inhibitors were analysed. The WT treated with Smo inhibitor were used to induce a systemic downregulation in Hh signalling. Between the Obese WT and Obese Gli3+/-, there was a significant difference in weight gain and liver/weight percentage, suggesting that Shh and/or Gli3 may play a role in the obese status in these animals. Obese WT animals gained weight more than Obese Gli3+/- and had higher liver/weight percentage, however, the Gli3+/- animals appeared to have worse glucose status and elevated Ly6G+ population. This could suggest that an increase in Shh is correlated with worse insulin sensitivity. As the deletion of one Gli3 allele is systemic, this finding could be caused be by several factors, including an alteration in the pancreas or the adipose tissue. Additionally, it is important to note that Hh functioning through Smo involves primary cilia. However, HSC, immune cells and hepatocytes do not have primary cilia, so the underlying mechanism may be through non-canonical pathway or involve other components (Grzelak et al., 2014, Yuan et al., 2015). Further research is needed for more in-depth investigation.

In the liver, macrophages are the largest population of mononuclear phagocytes, and are often associated with the severity of NAFLD (Wenfeng et al., 2014). The total hepatic macrophages were marked by $CD45^+Ly6G^-Nk1.1^-$

CD3⁻B220⁻CD11B⁺F4/80⁺ (Ramachandran et al., 2012). When Obese WT and Obese Gli3+/- mice were compared, there were no observed differences in the macrophage population. According to different publications, macrophages may be classified differently. Some studies divide the macrophages into either M1 or M2 macrophages, so that during liver injury and diseases, monocytes rapidly infiltrate into the liver sinusoids and mature (Heymann and Tacke, 2016). Macrophage can be polarised to become either M1 macrophages, which are classically activated, or M2 macrophages, which are activated through the alternative pathway (Italiani and Boraschi, 2014). Yet, others have proposed that macrophages have a spectrum of activation and Ly6C^{hi} are recruited due to inflammation and Ly6C^{low} are monocytes involved in patrolling (Mosser and Edwards, 2008). When Obese WT and obese Gli3+/- mice were compared, there appeared to be no statistical differences for the Ly6C+ macrophages. Comparing Obese WT treated with Smo inhibitors, and the obese WT without treatment, the ones which had the treatment had a decreased Ly6C+ macrophage population, suggesting that inhibition of Smo ameliorates the inflammation.

Normal healthy adult livers should not express Hh proteins, and the expression of Hh ligands is associated with the level of injury. However, it is important to note that adult models NAFLD with an association with Hh mechanism do not draw an inference to developmental programming, as in principle, the mechanism could be different during early development and adult. In young individuals, Hh is there to promotes organogenesis and

growth, whereas in adults, the mechanism is for healing wounds caused by either acute or chronic insults (Yao and Chuang, 2015). Another factor that needs to be taken in consideration is the method involved in this study. For the staining of the adult livers, potentially there could be interactions between anti-F4/80 and anti-GR-1 antibodies, leading to poor discrimination of F4/80 positive and negative cells within the CD11B cell population. However, it has been shown that pre-incubation with Gr-1, Ly6C or Ly6G antibodies prior to addition of F4/80 may correct this problem (Rose et al., 2012). This may be due to sharing of a similar epitope between the different anti-bodies.

Interestingly, the B cell population was elevated in the mice treated with Smo inhibitors. Presently, the role of B cells in NAFLD remains unclear. It has been observed that there is an increase in B cell activating factor in NAFLD patients (Zhang et al., 2016). Also, the severity of liver fibrosis in NAFLD correlates with Immunoglobulin A which is mainly produced by B cells (McPherson et al., 2014). One study has shown that B cell deficiency impedes the onset of immune response, with the possible explanation that B cells can act as antigen presenting cells for T cells (Bruzzi et al., 2018). Therefore, in this study the obese mice treated mice shown to have a profile of increased B cell population, could be early sign of immune activation and immune cell recruitment. However, the existing evidence remains inconclusive, as the change in B cells could be a systemic effect.

5.5. Conclusion

This chapter demonstrates that Hh is indeed involved in obesity and NAFLD. A reduction in Gli3 may lower the body mass, but it does not indicate better liver or diabetes status and immune profile. This could be since the animals have been on the obesogenic diet for prolonged period, that all the inflammatory indicators have been driven to the maximum. However, inhibition of Hh activation with Smo inhibitors ameliorates the inflammatory immune profile of NAFLD, yet interestingly without changes in weight or glucose status.

Chapter 6: The Hedgehog Pathway—the missing link between immunity, developmental programming and NAFLD?

6.1 Introduction

Hh is involved in the modulation of haematopoiesis. Many studies have focused on the impact of Hh on the adaptive immune system, particularly the influence of Hh on T cell differentiation in the thymus and B cell differentiation in the foetal liver (Hager-Theodorides et al., 2005, Solanki et al., 2017, Solanki et al., 2018).

Gli3+/- mice are mutant mice from Jackson's lab. They are heterozygous mice, with a mutation in one allele at the Gli3 locus. In many tissues, including the foetal liver, Gli3 gene acts mainly as a suppressor of the Shh pathway, so that a deficiency in Gli3 is accompanied by an increased in Shh signalling (Solanki et al., 2017). So, in this part of the project, Gli3+/- is used as a model of increased Shh expression, whereas Shh+/- is used as the opposite, a reduction of Shh.

This Chapter aims to build on the previous results chapters, which demonstrated that maternal obesity affects the development of foetuses and the foetal immune system and aims to investigate whether maternal diet interacts with the genetic makeup of foetuses. The **aim is to** investigate whether there are interactions between the maternal high fat diet and the Hh signalling pathway.

6.2. Method

Two animal phenotypes were used for this project: Wild Type (WT, female C57BL/6, Charles River Laboratories, UK), and Gli3+/- purchased from Jackson Laboratories. Gli3+/- mice were on pure C57BL/6 background and were genotyped by PCR. The feeding protocol was based on previous studies from our group (Samuelsson et al., 2008). Both WT and Gli3+/- were fed standard chow RMI (4% simple sugars, 3% fat, 50% polysaccharide, 15% protein [w/w], Special Dietary Services, energy 3.5 kcal/g) *ad libitum.* At around 10 weeks old, half of each experimental group were switched to a semi-synthetic energy-rich, high palatable obesogenic diet (10% simple sugars, 20% animal lard, 28% polysaccharide, 23% protein [w/w], Special Dietary Services, energy 4.5 kcal/g) supplemented *ad libitum* with sweetened condensed milk (approximately 55% simple sugar, 8% fat, 8% protein, w/w, nestle) and missed with mineral mix (AIN93G, Special dietary Services, 125 mg/pot) Mice then entered into the time-mating protocol after 8 weeks on their respective diet. After 8 weeks on their respective diets, time-mating was performed by mating a male with two females and monitoring the females for plugs. The day the plug is found was designated Embryonic Day 0.5 (E 0.5). Dams failing to become pregnant after development of a copulation plug were allowed to re-mate up to the age of 6 months. All animals were treated in accordance with the Animals (Scientific Procedures) Act, 1986 guidelines.

6.3. Results

6.3.1 The Gli3+/- mothers fed onto the HFD gained weight more rapidly.

In this experiment, female mice were on Gli3+/- background or control WT. Their control or experimental diet started at nine weeks old, with their weekly weight change recorded. It was observed that the Gli3+/- mice fed onto HFD gained weight more rapidly than the WT mice fed onto HFD. Whereas the WT mice and Gli3+/- mice fed on the control diet had similar weight gain **(Figure 32).**

6.3.2 Genetic background and maternal dietary status both exert influence on the foetal offspring body mass, but an interaction was not detected.

At E17.5, maternal obesity impacts the development of the foetuses, such that regardless of the genetic background, all the foetuses subjected to maternal obesity perinatally have smaller body mass compared to offspring of their lean counter parts **(Figure 33)**. This observation is consistent with the findings detailed in Chapter 3 of this thesis. The genetic background of each individual foetuses also impacts on the body mass, such that Gli3-/- foetuses are bigger than WT and Gli3+/- foetuses in the obese group; and in the control group there appears to be an upward trend from WT, to Gli3+/- to

Gli3-/-. These observations are consistent with the observations from Chapter 4, that mutation of the Gli3 gene increased the size of the foetus. Two-way ANOVA reveals that genetic influence and maternal diet impact on the foetal body mass separately, but there appears to be no interaction between the two factors. Multi comparison test with post hoc Tukey analysis shows statistical differences among the following pair of comparisons: WT_Ob vs Gli3+/-_Con; WT_Ob vs Gli3-/-_Con; WT_Ob vs Gli3-/-_Ob; Gli3+/-_Con vs Gli3+/-_Ob; Gli3+/-_Ob vs Gli3-/-_Con **(Figure 33)**.

For the placental mass, the results show no statistical differences among different groups. Neither the genetic background or the perinatal maternal diet exerted any detectable effects **(Figure 34).**

6.3.3. Genetic background and maternal dietary status influence the immune profile of the foetuses, and the two factors seem to interact.

Flow cytometry analysis of the foetal livers from the different experimental groups showed that for the neutrophil population, GR1+LY6G+, both the maternal dietary status and the genetic background of the foetuses altered the immune profile of the foetal livers **(Figure 35)**. There also appeared to be an interaction between the two factors. Multi comparison test reveals differences among the following pairs: WT_Con vs Gli3+/-_Ob; WT_Con vs Gli3-/-_Ob; WT_Ob vs Gli3+/-_Ob; WT_Ob vs Gli3-/-_Ob; Gli3+/-_Con vs

Gli3+/-_Ob; Gli3+/-_Con vs Gli3-/-_Ob; Gli3+/-_Ob vs Gli3-/-_Con; Gli3+/-_Ob vs Gli3-/-_Ob. The results show that a reduction in the Gli3 gene elevated the GR1+LY6G+ population in the foetal liver, and this population of cells appeared to be even more increased by maternal obesity **(Figure 35 A and B)**.

For the eosinophil population, GR1+LY6G-, the two-way ANOVA analyses revealed that maternal dietary status exerted an effect on the foetuses at E17.5, similar to the findings in Chapter 3 of the thesis. However, the genotype of the foetuses did not appear to have an effect. Yet, when taken together, the genetic background and the maternal dietary status seemed to interact with each other **(Figure 35 A and C).** The multi comparison test showed statistical differences between the following experimental groups: WT_Ob vs Gli3-/-_Con; WT_Ob vs Gli3-/-_Ob; Gli3+/-_Con vs Gli3-/-_Con; Gli3+/-_Con vs Gli3-/-_Ob. The general trend appears that a reduction in the Gli3 gene increased the percentage of the GR1+LY6G- population, and the maternal obesity exacerbated the effect further.

Interestingly, when the immune profile is analysed with GR1 against SSC, the pattern in the experimental groups is different from the results when analysed with GR1 against LY6G **(Figure 36)**. In the control groups, where the mothers were on the standard diet, the general trend is that the lack of Gli3 gene increase the GR1+ population, such that both Gli3+/-_Con and

Gli3-/-_Con have higher cell percentage than WT_Con. However, in the obese groups, where the mothers were fed with a HFD, the trend appeared to be reversed, despite not showing statistical differences among WT_Ob, Gli3+/-_Ob and Gli3-/-_Ob. Two-way ANOVA analyses reveal the genotype of the foetuses did not exert an effect on the variation, whereas the maternal diet influences the immune cell population. There also appears to be an interaction between the genotype and the maternal diet. Multiple comparison tests showed difference between the following pair of comparisons: WT_Con vs Gli3+/-_Con; WT_Con vs Gli3-/-_Ob; WT_Ob vs Gli3+/-_Con; WT_Ob vs Gli3-/-_Con; Gli3+/-_con vs Gli3+/-_Ob; Gli3+/-_Con vs Gli3-/-_Ob; Gli3-/-_Ob vs Gli3-/-_Ob **(Figure 36)**.

For macrophages, marked by F4/80 or CD11B antibodies, both the genotype of the foetuses and the maternal dietary status have an influence. However, two-way ANOVA analyses suggest no interaction between the two factors. Also, within the control groups, there is no significant difference among the three groups (WT_Con, Gli3+/-_Con and Gli3-/-_Con). For the obese group, Gli3-/-_Ob has the least percentage of macrophages and is statistically different from all other five experimental groups (WT_Con; Gli3+/-_Con; Gli3-/-_Con, WT_Ob; Gli3+/-_Ob) **(Figure 37)**.

Figure 32. Weight change of mothers before pregnancy
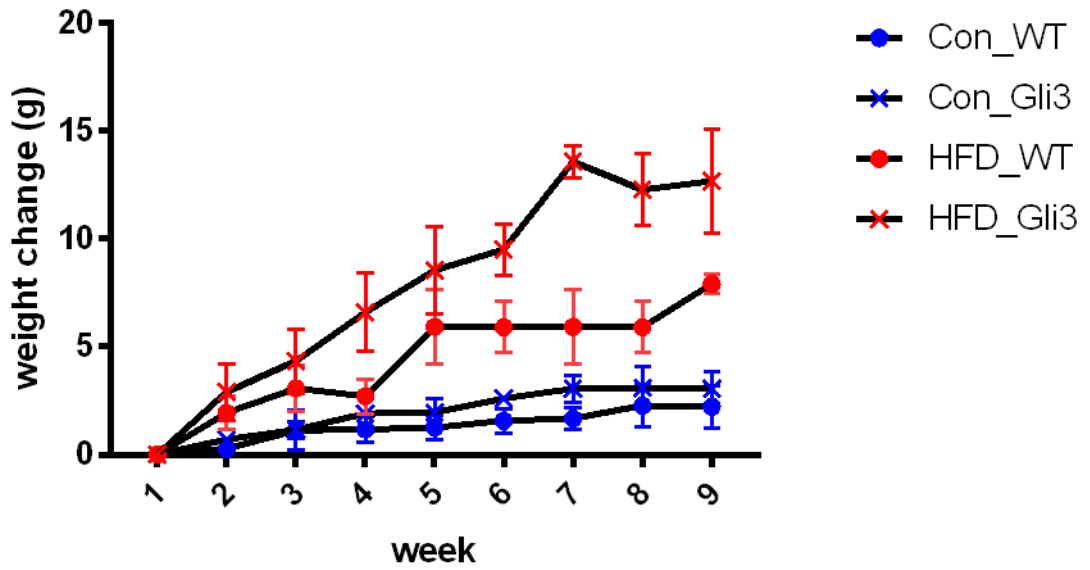
Figure 32: Weight change of WT and Gli3+/- mothers before pregnancy over eight weeks. Each line represents different dietary and genetic background combinations.

p<0.05, n=8, error bar±SEM

Figure 33. Foetal weight at E17.5
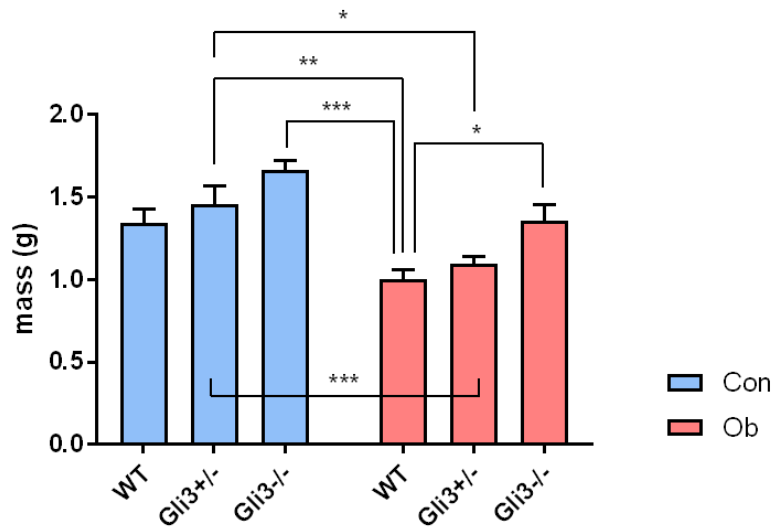
Comparison

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	0.3413	-0.008831 to 0.6915	No	ns	0.0585
WT:Con vs. Gli3+/-:Con	-0.1167	-0.5082 to 0.2748	No	ns	0.9285
WT:Con vs. Gli3+/-:Ob	0.2473	-0.1028 to 0.5975	No	ns	0.2661
WT:Con vs. Gli3-/-:Con	-0.3233	-0.7148 to 0.06816	No	ns	0.1416
WT:Con vs. Gli3-/-:Ob	-0.01667	-0.3668 to 0.3335	No	ns	>0.9999
WT:Ob vs. Gli3+/-:Con	-0.458	-0.8082 to -0.1078	Yes	**	0.0066
WT:Ob vs. Gli3+/-:Ob	-0.094	-0.3973 to 0.2093	No	ns	0.9168
WT:Ob vs. Gli3-/-:Con	-0.6647	-1.015 to -0.3145	Yes	***	0.0001
WT:Ob vs. Gli3-/-:Ob	-0.358	-0.6613 to -0.05475	Yes	*	0.0155
Gli3+/-:Con vs. Gli3+/-:Ob	0.364	0.01384 to 0.7142	Yes	*	0.0389
Gli3+/-:Con vs. Gli3-/-:Con	-0.2067	-0.5982 to 0.1848	No	ns	0.5621
Gli3+/-:Con vs. Gli3-/-:Ob	0.1	-0.2502 to 0.4502	No	ns	0.9395
Gli3+/-:Ob vs. Gli3-/-:Con	-0.5707	-0.9208 to -0.2205	Yes	***	0.0008
Gli3+/-:Ob vs. Gli3-/-:Ob	-0.264	-0.5673 to 0.03925	No	ns	0.1097
Gli3-/-:Con vs. Gli3-/-:Ob	0.3067	-0.0435 to 0.6568	No	ns	0.1064

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	0.201	0.9339	ns	No
diet	29.34	0.0012	**	Yes
genotype	41.16	<0.0001	****	Yes

Figure 33: Body mass of WT, Gli3+/- and Gli3-/- foetuses subjected to maternal obesity compared to controls at E17.5. *p<0.05, **p<0.01, *<0.001, n=15, bar±SEM.**
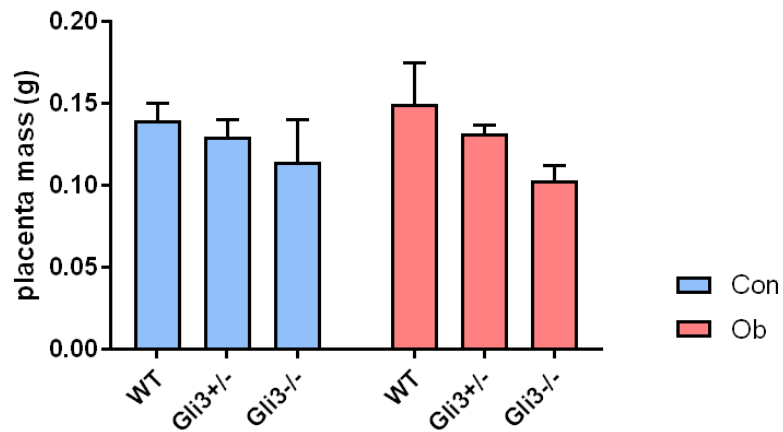
Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	-0.01	-0.08797 to 0.06797	No	ns	0.998
WT:Con vs. Gli3+/-:Con	0.01	-0.07717 to 0.09717	No	ns	0.9989
WT:Con vs. Gli3+/-:Ob	0.008	-0.06997 to 0.08597	No	ns	0.9993
WT:Con vs. Gli3-/-:Con	0.025	-0.07246 to 0.1225	No	ns	0.9564
WT:Con vs. Gli3-/-:Ob	0.03667	-0.0505 to 0.1238	No	ns	0.7451
WT:Ob vs. Gli3+/-:Con	0.02	-0.05797 to 0.09797	No	ns	0.9564
WT:Ob vs. Gli3+/-:Ob	0.018	-0.04952 to 0.08552	No	ns	0.949
WT:Ob vs. Gli3-/-:Con	0.035	-0.05432 to 0.1243	No	ns	0.7946
WT:Ob vs. Gli3-/-:Ob	0.04667	-0.0313 to 0.1246	No	ns	0.4148
Gli3+/-:Con vs. Gli3+/-:Ob	-0.002	-0.07997 to 0.07597	No	ns	>0.9999
Gli3+/-:Con vs. Gli3-/-:Con	0.015	-0.08246 to 0.1125	No	ns	0.9954
Gli3+/-:Con vs. Gli3-/-:Ob	0.02667	-0.0605 to 0.1138	No	ns	0.9129
Gli3+/-:Ob vs. Gli3-/-:Con	0.017	-0.07232 to 0.1063	No	ns	0.9879
Gli3+/-:Ob vs. Gli3-/-:Ob	0.02867	-0.0493 to 0.1066	No	ns	0.8328
Gli3-/-:Con vs. Gli3-/-:Ob	0.01167	-0.08579 to 0.1091	No	ns	0.9986

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	1.632	0.8543	ns	No
diet	17.85	0.2091	ns	No
genotype	0.0002777	0.9942	ns	No

Figure 34: placental mass of WT, Gli3+/- and Gli3-/- foetuses subjected to maternal obesity compared to controls at E17.5, n=15, bar±SEM.

Figure 35

A. Neutrophil and eosinophil cell population
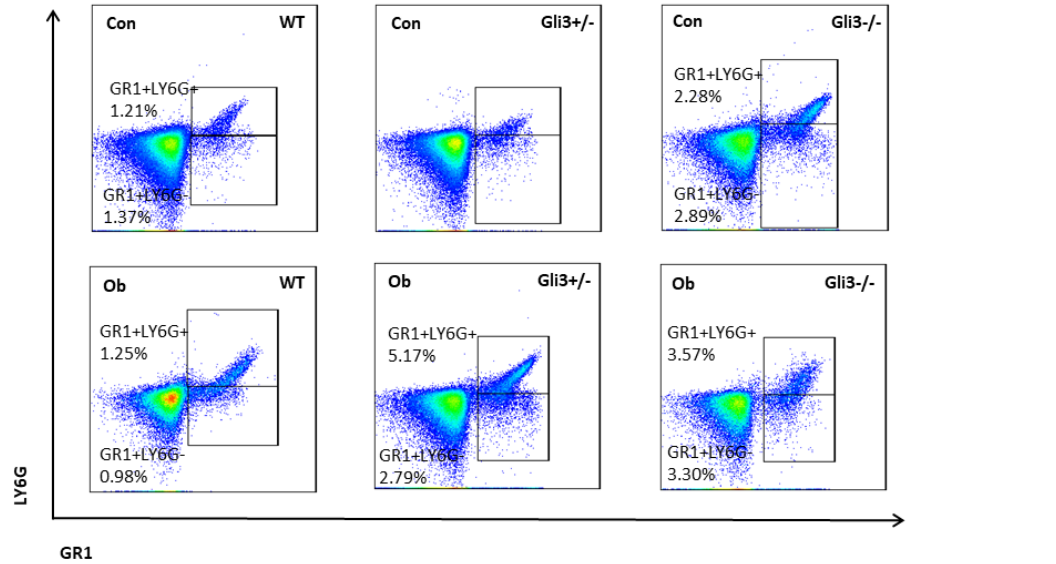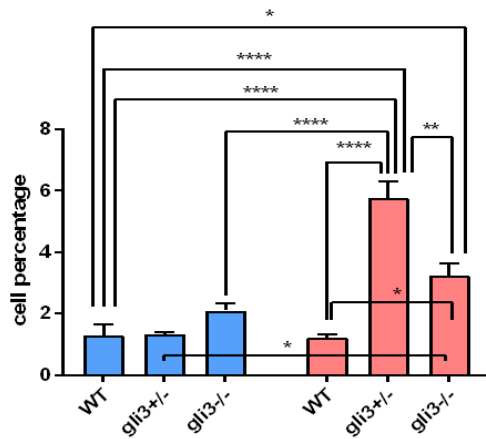
B. Neutrophil cell percentage

C. Eosinophil cell percentage
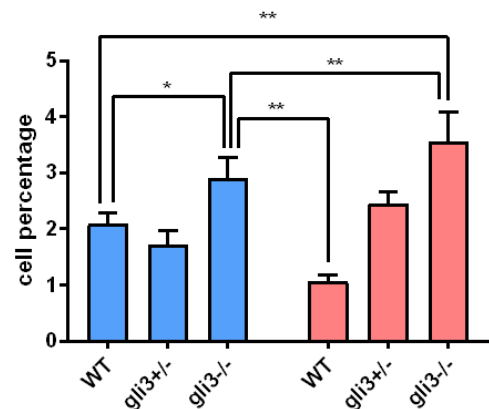
Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	29.46	<0.0001	****	Yes
Genotype	29.13	<0.0001	****	Yes
Maternal diet	24.16	<0.0001	****	Yes

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	14.42	0.0337	*	Yes
Genotype	43.8	0.0003	***	Yes
Maternal diet	0.3621	0.6605	ns	No

Neutrophil cell percentage comparison

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	0.05114	-1.34 to 1.442	No	ns	>0.9999
WT:Con vs. gli3+/-:Con	-0.05286	-1.322 to 1.217	No	ns	>0.9999
WT:Con vs. gli3+/-:Ob	-4.496	-5.818 to -3.175	Yes	****	<0.0001
WT:Con vs. gli3-/-:Con	-0.8271	-2.097 to 0.4423	No	ns	0.3741
WT:Con vs. gli3-/-:Ob	-1.97	-3.608 to -0.3306	Yes	*	0.0115
WT:Ob vs. gli3+/-:Con	-0.104	-1.495 to 1.287	No	ns	>0.9999
WT:Ob vs. gli3+/-:Ob	-4.547	-5.985 to -3.109	Yes	****	<0.0001
WT:Ob vs. gli3-/-:Con	-0.8783	-2.269 to 0.5124	No	ns	0.4079
WT:Ob vs. gli3-/-:Ob	-2.021	-3.755 to -0.2862	Yes	*	0.0152
gli3+/-:Con vs. gli3+/-:Ob	-4.443	-5.765 to -3.122	Yes	****	<0.0001
gli3+/-:Con vs. gli3-/-:Con	-0.7743	-2.044 to 0.4952	No	ns	0.4459
gli3+/-:Con vs. gli3-/-:Ob	-1.917	-3.556 to -0.2778	Yes	*	0.0147
gli3+/-:Ob vs. gli3-/-:Con	3.669	2.348 to 4.99	Yes	****	<0.0001
gli3+/-:Ob vs. gli3-/-:Ob	2.527	0.8473 to 4.206	Yes	**	0.001
gli3-/-:Con vs. gli3-/-:Ob	-1.142	-2.781 to 0.4965	No	ns	0.3031

Eosinophil cell percentage comparison

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	1.027	-0.6709 to 2.724	No	ns	0.4404
WT:Con vs. gli3+/-:Con	0.3695	-1.065 to 1.804	No	ns	0.9648
WT:Con vs. gli3+/-:Ob	-0.3648	-1.799 to 1.07	No	ns	0.9667
WT:Con vs. gli3-/-:Con	-0.8183	-2.288 to 0.6518	No	ns	0.5288
WT:Con vs. gli3-/-:Ob	-1.47	-3.168 to 0.2276	No	ns	0.1165
WT:Ob vs. gli3+/-:Con	-0.6571	-2.092 to 0.7776	No	ns	0.7143
WT:Ob vs. gli3+/-:Ob	-1.391	-2.826 to 0.0433	No	ns	0.0609
WT:Ob vs. gli3-/-:Con	-1.845	-3.315 to -0.3748	Yes	**	0.0084
WT:Ob vs. gli3-/-:Ob	-2.497	-4.194 to -0.7991	Yes	**	0.0017
gli3+/-:Con vs. gli3+/-:Ob	-0.7343	-1.846 to 0.377	No	ns	0.3464
gli3+/-:Con vs. gli3-/-:Con	-1.188	-2.345 to -0.03114	Yes	*	0.0418
gli3+/-:Con vs. gli3-/-:Ob	-1.84	-3.274 to -0.4048	Yes	**	0.0069
gli3+/-:Ob vs. gli3-/-:Con	-0.4536	-1.61 to 0.7031	No	ns	0.8243
gli3+/-:Ob vs. gli3-/-:Ob	-1.105	-2.54 to 0.3295	No	ns	0.2008
gli3-/-:Con vs. gli3-/-:Ob	-0.6517	-2.122 to 0.8185	No	ns	0.7406

Figure 35: The immune profile of WT, Gli3+/-, Gli3-/- foetuses subjected to maternal obesity compared to controls at E17.5.

(A) flow cytometry diagram showing GR1+ and/or LY6G+ population; (B) bar chart showing GR1+LY6G+ cell population in different groups; (C) bar chart showing GR1+LY6G- population in different groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=7, bar±SEM.

A. Granulocytes

B. Granulocyte cell percentage

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	14.88	0.0019	**	Yes
Genotype	3.276	0.2013	ns	No
Maternal diet	54.27	<0.0001	****	Yes

Granulocyte cell percentage comparison

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	1.057	-1.583 to 3.696	No	ns	0.8275
WT:Con vs. gli3+/-:Con	-2.793	-5.433 to -0.1536	Yes	*	0.0331
WT:Con vs. gli3+/-:Ob	1.931	-0.6047 to 4.468	No	ns	0.2209
WT:Con vs. gli3-/-:Con	-2.263	-4.799 to 0.2733	No	ns	0.1025
WT:Con vs. gli3-/-:Ob	3.436	0.6578 to 6.214	Yes	**	0.0085
WT:Ob vs. gli3+/-:Con	-3.85	-6.589 to -1.111	Yes	**	0.0022
WT:Ob vs. gli3+/-:Ob	0.8748	-1.765 to 3.514	No	ns	0.9133
WT:Ob vs. gli3-/-:Con	-3.32	-5.959 to -0.6798	Yes	**	0.0072
WT:Ob vs. gli3-/-:Ob	2.379	-0.4937 to 5.252	No	ns	0.1517
gli3+/-:Con vs. gli3+/-:Ob	4.725	2.085 to 7.364	Yes	****	<0.0001
gli3+/-:Con vs. gli3-/-:Con	0.5305	-2.109 to 3.17	No	ns	0.9896
gli3+/-:Con vs. gli3-/-:Ob	6.229	3.356 to 9.102	Yes	****	<0.0001
gli3+/-:Ob vs. gli3-/-:Con	-4.194	-6.73 to -1.658	Yes	***	0.0003
gli3+/-:Ob vs. gli3-/-:Ob	1.505	-1.274 to 4.283	No	ns	0.5791
gli3-/-:Con vs. gli3-/-:Ob	5.699	2.921 to 8.477	Yes	****	<0.0001

Figure 36: The immune profile of WT, Gli3+/-, Gli3-/- foetuses subjected to maternal obesity compared to controls at E17.5.

(A) flow cytometric diagram showing GR1+ population from different groups;

(B) bar chart showing GR1+ cell population from different groups. *p<0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=7.

A. Macrophage

Ob — WT — 3.83%
Ob — Gli3+/- — 4.83%
Ob — Gli3-/- — 1.25%

F4/80

CD11B

B. Macrophage percentage

cell percentage

****
***
**
**
***

WT gli3+/- gli3-/- WT gli3+/- gli3-/-

Con
Ob

Source of Variation	% of total variation	P value	P value summary	Significant?
Interaction	6.897	0.0862	ns	No
Genotype	34.1	<0.0001	****	Yes
Maternal diet	19.53	0.0005	***	Yes

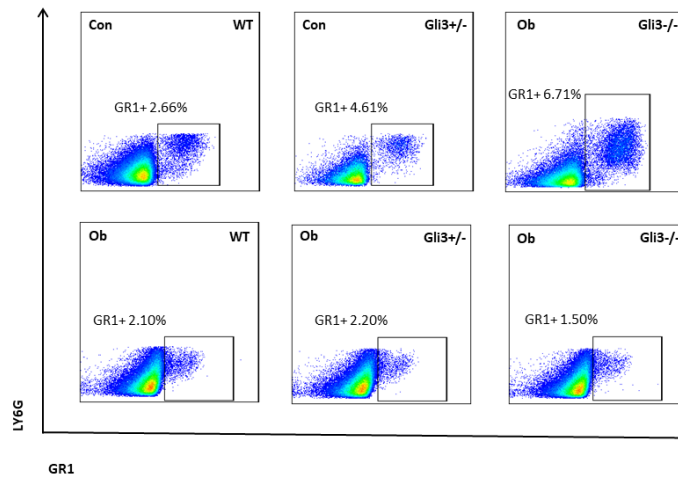## C. Macrophage percentage comparison

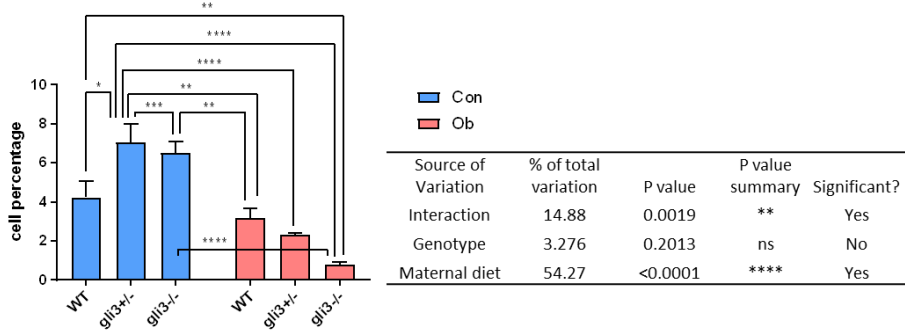| Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
WT:Con vs. WT:Ob	1.649	-0.4457 to 3.743	No	ns	0.1926
WT:Con vs. gli3+/-:Con	0.9214	-1.173 to 3.016	No	ns	0.7664
WT:Con vs. gli3+/-:Ob	1.256	-0.8386 to 3.35	No	ns	0.4717
WT:Con vs. gli3-/-:Con	1.754	-0.34 to 3.849	No	ns	0.1439
WT:Con vs. gli3-/-:Ob	3.971	1.876 to 6.064	Yes	****	<0.0001
WT:Ob vs. gli3+/-:Con	-0.7271	-2.513 to 1.059	No	ns	0.8186
WT:Ob vs. gli3+/-:Ob	-0.3929	-2.179 to 1.393	No	ns	0.9846
WT:Ob vs. gli3-/-:Con	0.1057	-1.68 to 1.892	No	ns	>0.9999
WT:Ob vs. gli3-/-:Ob	2.321	0.5354 to 4.107	Yes	**	0.0051
gli3+/-:Con vs. gli3+/-:Ob	0.3343	-1.452 to 2.12	No	ns	0.9926
gli3+/-:Con vs. gli3-/-:Con	0.8329	-0.9532 to 2.619	No	ns	0.7209
gli3+/-:Con vs. gli3-/-:Ob	3.049	1.263 to 4.835	Yes	***	0.0002
gli3+/-:Ob vs. gli3-/-:Con	0.4986	-1.287 to 2.285	No	ns	0.9568
gli3+/-:Ob vs. gli3-/-:Ob	2.714	0.9283 to 4.5	Yes	***	0.0008
gli3-/-:Con vs. gli3-/-:Ob	2.216	0.4297 to 4.002	Yes	**	0.0082

Figure 37: The immune profile of WT, Gli3+/-, Gli3-/- foetuses subjected to maternal obesity compared to controls at E17.5.

(A) flow cytometric diagram showing macrophage population from different groups; (B) bar chart showing macrophage population from different groups. *p<0.05, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=7, bar±SEM.

6.4. Discussion

6.4.1. Relationship between maternal obesity and foetal body mass

The current study has clearly shown that embryos from obese mothers have lower body mass at E17.5. In comparison to humans, many studies have suggested that maternal obesity is more likely to lead to babies that are large for gestational age (LGA) with macrosomia (Gaudet et al., 2014, Marchi et al., 2015). Only a small number of studies have reported cases of small for gestational age (SGA) babies born to obese mothers. Catov et al reported in a cohort study that both LGA and SGA occur from obese pregnant mothers, with rate of occurrence for SGA higher than LGA. This paradoxical relationship may be explained by how quickly the pregnant mothers gain weight and the precise gestational period the pregnant mothers gain weight; with fast and early gestational weight gain leading to LGA, and late and slow weight gain leading to SGA (Catov et al., 2015). The contradiction of observations suggests that obesity during pregnancy certainly leads to abnormal foetal growth, and the resulting LGA or SGA may be due to different mechanisms.

The results of the current study may be consistent with the 'thrifty hypothesis' proposed by Barker, which suggests that early reduced foetal growth predisposes for chronic health conditions. Effectively, maternal obesity could have induced a starvation environment to the embryos, leading to the embryos becoming smaller than the control embryos, thus these offspring become

unable to cope with the energy dense environment postnatally (Barker and Osmond, 1986a, Hales et al., 1991). This finding is particularly interesting and relevant to clinical practice. Many pregnant women are under the assumption that during pregnancy they should consume more calories for the foetus. Yet the current study suggests that excessive increase in body weight during pregnancy, in fact, hinders the normal development of foetuses.

6.4.2 Maternal obesity and foetal hepatic immunity

Liver organogenesis starts at E8.4 in mice, formed from ventral foregut. The foetal liver is colonised by hematopoietic progenitors and transiently becomes the principal and primary hematopoietic organ. Therefore, the foetal liver has a crucial role in shaping long-term immunity.

Our current results showed that the immune profile in the foetal liver is altered in the embryos from obese mothers, with an increase in neutrophils in wild-type embryos from obese mothers compared to wild-type embryos from control mothers. Neutrophil status has long been associated with fatty liver disease. NASH is accompanied by an accumulation of neutrophils, and neutrophil dysfunction is associated with liver fibrosis and cirrhosis and has been suggested to induce the activation of HSCs (Xu et al., 2014). Some human biopsy studies have even suggested that neutrophil to lymphocyte ratio (NLR) could be a biological marker for the presence of steatohepatitis (Abdel-Razik

et al., 2016), although there also exists the opposing argument that NLR is not a reliable indicator for NAFLD (Kara et al., 2015) . However, presently, no studies have observed an alteration of liver neutrophil profile in embryos exposed to maternal obesity. Therefore, this study is the first to suggest that maternal obesity modulates the development of neutrophils, which may play a role in the immune mechanism of NAFLD pathogenesis. Although, eosinophils are from the same lineage as neutrophils, they were not affected by the genetic background of the foetuses but were increased by maternal obesity. Additionally, there was also an interaction between maternal obesity and the genotype of the foetuses. The increase in eosinophil numbers is in line with findings from other studies. It has been shown in humans that genes associated with eosinophil function are upregulated in NASH.

The macrophage profile was affected by maternal obesity and genotype separately, without interaction between the two factors. Mouralidarane et al had reported that maternal obesity increased the number of macrophages in the liver but decreased their phagocytic function (Mouralidarane et al., 2013). This implies that the programming effect on macrophages might take place postnatally and could be due to the influence of breast milk during the lactation period. Evidence has suggested that leptin, which is abundant in the breast milk of obese mothers, plays an immunological role (Naylor and Petri, 2016). In addition, a recent study has reported an interactive relationship between leptin and macrophage *in vitro* (Shivahare et al., 2015).

Alternatively, another prevailing theory on the pathogenesis of NAFLD may explain the maternal programming of macrophages. NAFLD is associated with dysbiosis of gut microbiota, with an alteration in the Firmicutes to Bacteroidetes ratio (Frasinariu et al., 2013). Consequently, the microbiota composition may shape the immune system of an individual. It is thought that embryos are sterile inter-uterine, and offspring develop microbiota composition resembling their mothers' gut postnatally (Frazier et al., 2011). Therefore, one possibility is that maternal obesity alters the microbiota of the mother, which in turn impacts on the gut flora in offspring, thus affecting the development of hepatic macrophages.

6.4.3. Interaction between maternal obesity and the Hh pathway

Hh signalling appears to interact with maternal diet in altering foetal liver development. Embryos from Gli3+/- lean mothers exhibited a significant difference compared to embryos from control lean mothers in their neutrophil and NK cell populations. Although there is literature evidence that T cell development is affected by the Hh pathway in foetal liver and foetal thymus (Solanki et al., 2017, Solanki et al., 2018, Hager-Theodorides et al., 2005), limited evidence exists to suggest that Hh also influences the development of granulocytes (Lau et al., 2012). Some studies have highlighted the relationship between Hh and NK cells (Choi and Diehl, 2005). One study showed that exposure of Shh ligands promotes NKT cell viability and proliferation, leading to more severe fibrosis in the liver (Choi et al., 2010). Clearly, our results

suggest that Hh modulates development of the innate immune cells. However, the combination of maternal obesity and genetic alteration reversed the difference, suggesting that maternal obesity may be interacting with the Hh pathway.

6.5. Conclusion

Both genotype and maternal diet have an impact on the immune profile of the foetal livers and there appears to be an interactive relationship between the two factors. The exact mechanism involved requires further investigation.

Chapter 7: Discussion & Conclusion

This project forms a continuation of previous works, to investigate the influence of maternal diet at an earlier time point of development, which is also arguably the most plastic period of development. It focuses on asking four questions：the first question is how early maternal obesity influences the offspring and what mechanism or pathway is involved in the process. Hedgehog signalling pathway was chosen as the candidate, due to its close association to both NAFLD, immune development and organ development. Thus, the general hypothesis that there is an interaction between the maternal dietary status and the Hh pathway. This was supported by studies highlighting the involvement of Hh in NAFLD, and particularly NAFLD induced liver fibrosis. To further validate these studies, this project then investigated the relationship between obesity, NAFLD and Hh, using Gli3+/-, Shh+/- and WT with Smo inhibitors as the animal models.

In summary, maternal obesity impacts on the offspring as early as the foetal stage, at E14.5 and E17.5. It was found that the maternal obesity caused the foetuses to be smaller than the controls and to have livers containing fewer cells. The immune profile from these foetal livers was also affected by maternal obesity, with decreased macrophage, decreased granulocyte, decreased NK cells, decreased CD3+ cells and decreased CD19+B220+ cells at E14.5; increased granulocyte, increased eosinophils and increased CD19+ B cells at E17.5. The changes are accompanied by altered gene expression of increased *Tgfβ* and increased *Gli3*. **Chapter 3**. An attempt was made to investigate the role of Hh in the observed phenomenon. The evidence from my experiments

showed that Hh has a role in modulating the development of the innate system with increased weight, decreased cell number, increased granulocytes, increased neutrophils and increased T cells in gli3 deficient foetuses. Whereas in shh deficient foetuses, the general trend is the opposite of gli3 deficient foetuses with decreased foetal weight, decreased cell number, decreased granulocytes and decreased T cells **Chapter 4**. To further investigate the relationship and possible involvement of Gli3 and Shh, the male adult mice were made obese, and it was observed that Gli3+/- male mice gain weight less rapidly than the WT mice, although the final weights were similar, and the immune profile of the livers from these mice were similar. However, for WT obese mice treated with Smo inhibitor, it was shown that the immune cell profile leans towards a less inflammatory status **Chapter 5.** Lastly, in **Chapter 6**, the results show that an alteration in Hh has an additive effect with perinatal maternal obesity, exacerbating the proinflammatory and activated immune profile. All these are interesting observations; however, the results are still inconclusive to pinpoint the exact mechanisms involved. Nevertheless, this thesis having the focus on the immune system, Hedgehog signalling pathway and NAFLD, it demonstrates the permanent remodelling of the livers at the foetal stage due to maternal obesity, and it is the first of its kind in attempting to link DP, immune system, Hedgehog and NAFLD **(Figure 38)**.
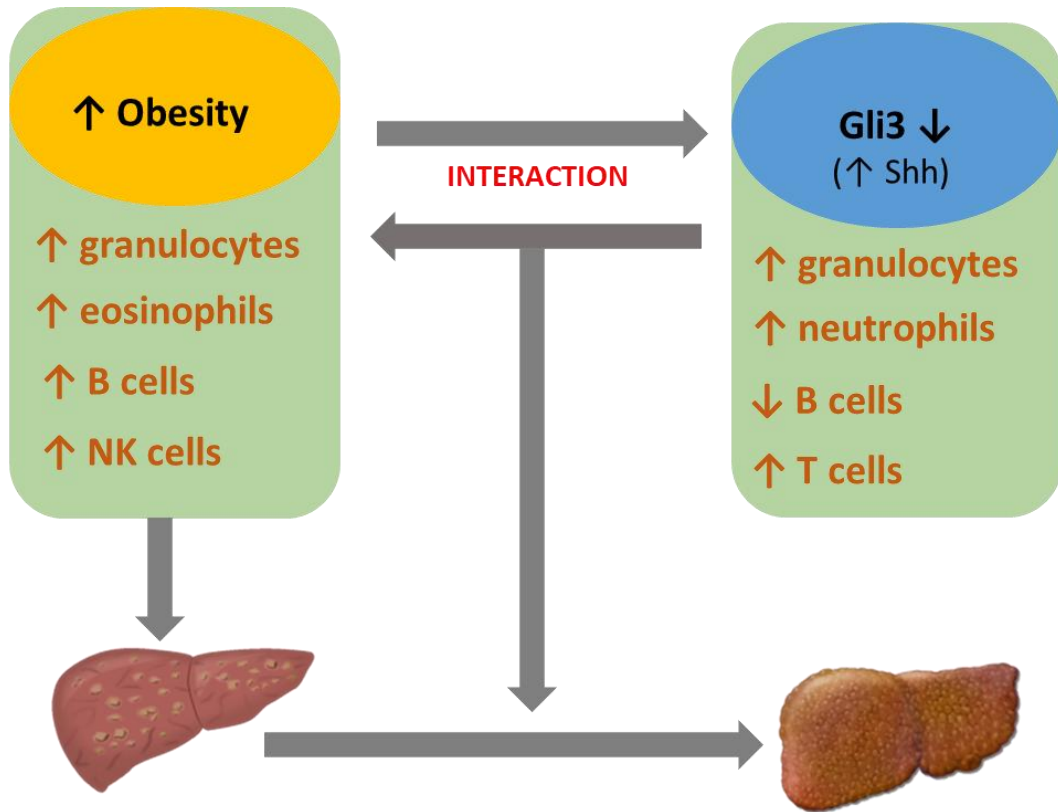
Figure 38: A summary of the interactive effect between maternal obesity and genotype of the foetuses.

7.1. Limitations

One limitation of this study was the use of animal models which have different physiology to the humans. Another limitation is that the genetically modified mouse model with mutant Gli3 gene had a global (constitutive) mutation rather than a conditioned mutation; therefore, it would be difficult to identify a tissue specific effect, in this case the liver. Part of the ongoing research is to use conditional knockout animals, so it would give a clearer idea on the interactive

relationship between maternal obesity and the genetic alteration specifically to liver.

There are certain limitations and areas which could be further improved. The purpose of the diet, 45% fat diet supplemented with 20% sugar from the condensed milk, is to provide a high sugar high fat diet, which mimics the human diet composition of the obese population. This is an effective diet, such that the mice gain weight rapidly, and in 8 weeks, the mice gained over 30% of their original body weight. Additionally, the diet is rather moderate for promoting weight gain, rather than aggressively encouraging weight gain. This is more in line with the chronic inflammatory status due to obesity. However, the limitations and short comes of the diet is that the setup is messy, and it is difficult to measure the food consumption level. Because there are two sources of energy, experimental diet and condensed milk. It is difficult to know from which source the mice obtain their food and changes in appetite, for example due to genetic background, could also influence experimental outcomes.

We should also take into consideration the number of animals housed in a cage for the experiment setup. It is known that depending on the total number of animals in the cage, the mice have different weight and food consumption, such that, a single housed mouse gains more weight than group housed. This phenomenon was quickly observed in the project, so all animals were standardised to be housed in pairs. However, it still does not eliminate other confounding factors completely. In the event that one of the mice died, or one

mouse was used for experiment, the other mouse was still single housed for a short period. Additionally, when housed in a pair, there is the social hierarchy within the groups which needed to be considered. The more dominant mouse tends to consume more food, thus has higher weight. This is less important for the experiments in results chapter 3 and experiments in results chapter 5. However, in **Chapter 4**, where the WT and Gli3+/- mice were housed together, this does not eliminate the possibility that WT gained more weight, because by nature WT tends to be more dominant compared to Gli3+/-. In this instance, we assumed that this social hierarchy did not play a role, but this should be taken into consideration when interpreting the results.

Another unpredictable nature of animals is the seasonal change. Although the mice were housed in a controlled environment, they could still somehow sense the season. It was observed that the fertility rate of the mice was at the highest in the Spring, and studies have shown that in the spring, the testicles of mice enlarge. As obesity is tied to metabolism which in turn is dependent on season, which may have influenced the outcome. There are certain hormonal changes, which could have a potential effect on the experimental readouts. Again, as the project was conducted in a random fashion, it was assumed all these confounding factors were eliminated. Taken together, animal models are effective tools in providing indications of the mechanism and allowing researchers to observe in-utero or long-term phenomenon in a controlled manner. However, due to certain restrictions: limited number of animals used, different physiology of mice and humans; all results should be interpreted

alongside human studies, and to complement with human studies.

There are some disadvantages in using the proposed experimental diet. Firstly, it is supposed to mimic the human obese diet. However, as it is a mixed diet, with pelleted food supplemented with high sugar in the form of condensed milk. It is not certain from which source of food the mice obtained their calorie intake from. Additionally, the experimental setup has the flaws that food intake could not be recorded. The HFD crumbled very easily because it was high in fat. As for the condensed milk, when the mice first subjected to the milk in the jar, it took them two to three days to acclimatise to eating from the small jar. All these variations with animal experiments should be taken into consideration.

7.2. Foetal growth restriction

In **Chapter 3, 4 and 6**, the results suggest that maternal obesity restricts the growth of foetuses. It would be interesting to further investigate the mechanism. There is evidence suggesting that placental restriction results in intrauterine growth restriction through the decreased gene expression of growth hormone (GH) receptor, prolactin receptor (RPLR), Insulin-like growth factor (IGF)-1 and IGF-2, with results coming from starvation models (Lasuncion et al., 1987). For this project which is a maternal obesity model, the placenta samples from this project can also be studied for these genes. Also, we can examine the immune profile of these placentas, as the maternal immune cells cross the placenta after gestation E16.5 (Arck and Hecher, 2013). Additionally, Challier et al showed two to three-fold increase in the number of placental macrophages in obese women (Challier et al., 2008).

7.3. Adult studies

For future work, there remain many questions to be asked. This thesis may have screened the immune system involved in the developmental programming mechanism of NAFLD. Yet, there could be more in-depth investigation into the genes involved in the observed changes. The observations from the adult study in **Chapter 5** are interesting for further investigation. Adipose tissue macrophage infiltration is accompanied by an

elevation in OPN. As OPN highly secreted by macrophages and also a target of Hh, this could be a gene of interest for further research.

7.4. Paternal influence

Many of the existing studies focus on maternal influence on the offspring. Yet, not many studies on the paternal effect. There is already some evidence from obese mice models utilising IVF, that gametes from obese paternal source worsens the obesity and glucose status.

7.5. Microbiota

This thesis focused on examining the foetal livers, at the stage of haematopoiesis, based on the hypothesis that Hh may be involved. Before the experiment, part of the research proposal was to investigate the immune mechanism by studying the potential interactions between the microbiota and developmental programming in the immune mechanism of NAFLD, by manipulating the maternal microbiota composition using antibiotics or germ-free animal model. However, due to limitations on the animal facilities and other resources, it was not possible to investigate this. Therefore, for the next part of the project, we would like to continue this topic and search for better understanding of the immune mechanism involved in developmental programming of NAFLD. We will investigate other factors which could

influence the immune mechanism. For instances, the involvement of breast milk and/or microbiota transfer between the mother and foetus, as there is evidence that human milk has been shown to contain bacteria, an important source for 'establishing' a healthy microbiome (Jost et al., 2015). The liver is positioned between the gut and the circulatory system, making it an important organ for local and systemic immune response. Additionally, the results from this thesis suggest that eosinophils in the foetal liver are affected by maternal obesity, and the interaction between maternal obesity and genotype, but not by genotype of the foetuses alone in the foetal liver, which are cells often associated with worms and parasites.

Therefore, for future work, we could purse the questions of how the maternal obesity shapes the foetal microbiota environment and whether this would lead to the alteration of the immune system observed in **Chapter 1.** Additionally, there are distinct microbiota composition in the placenta and meconium, indicating early signs of microbiota colonisation. This may be one of the explanations observed in **Chapter 1** of this thesis, that alteration in placenta and meconium may play a role in affecting the immune status of the foetuses.

The involvement of microbiota in obesity and NAFLD is a prevailing theory. The liver receives 25% blood from the hepatic artery and 75% from the gut via the portal veins. And most cells have receptors which are specific to the pathogens: toll-like receptors (TLR). Gordon et al stated that germ free mice had 40% less body fat than mice with a normal gut microbiota, even though

the latter ate 30% less calories. It has been also described that genetically obese ob/ob mice had a 50% reduction abundance of Bacteroides and a proportional increase Firmicutes compared to their lean counterparts. The theory of microbiome involvement rooted with the concept of dysbiosis. The disturbance would lead to abnormal accumulation of bacterial products passing through the hepatic portal vein. When the liver is conditioned by first hits, such as lipid accumulation, the further assaults from the endotoxins, TLR agonists and inflammasomes can progress into NAFLD. Further evidence for the microbiome, is that TLR2 and its agonists palmitic acid can activate inflammasome in Kupffer cells. Yet, TLR2 KO mice appeared to have ameliorated liver condition. Turnbaugh et al transplanted faecal microbiota from lean and ob/ob mice to germ-free wild-type. After 2 weeks, rodents hosting the microbiota from obese mice increased their fat mass and extracted more calories from food than the lean mice hosting the gut microbiota from lean mouse donors (Turnbaugh et al., 2006).

Taken together, these are the further steps I would like to take for future work:

1. Pin point the mechanisms involved in the observed adult studies by further gene and protein analyses.

2. Develop further understanding of the maternal influence and the changes observed in this thesis, by analysing further factors which have close relationship with the immune mechanism.

3. Investigate the paternal influence on the offspring livers and metabolic status.

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