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Maternal metformin treatment in obese pregnancy alters priming of offspring metabolic dysfunction and nonalcoholic fatty liver disease in mice

by

Hugh Thomas

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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MATERNAL METFORMIN TREATMENT IN OBESE PREGNANCY ALTERS PRIMING OF OFFSPRING METABOLIC DYSFUNCTION AND NONALCOHOLIC FATTY LIVER DISEASE IN MICE

Hugh Nicholas Belle Thomas

Background: Interventions to protect offspring from the deleterious long-term effects of maternal obesity during pregnancy have been sought but have proven largely ineffective in humans. Metformin, an antidiabetic drug used to control gestational diabetes, failed to reduce obesity-associated fetal overgrowth in two human trials, yet long-term follow-up of these children will take many years. Thus, this thesis aims to characterize the effect of metformin on offspring in a mouse model of maternal obesity.

Methods: Female mice were fed normal chow (C; 7% kcal fat) or obesogenic high-fat diet (HF; 45% kcal fat) for 6 weeks prior to mating. Metformin (m) was given in drinking water (250mg/kg) to half of dams during pregnancy and lactation. Dam and fetal parameters were assessed in a subgroup at gestation day 16.5. At weaning, offspring received C or HF diet. Offspring metabolic parameters were assessed at 28 weeks, and offspring were killed and tissue collected at 30 weeks. Plasma metabolic and inflammatory markers and hepatic gene expression, lipid levels and pathology were evaluated.

Results: In fetuses, metformin treatment altered fetal hepatic gene expression and reduced fetal weight. Metformin had no effect on obese dam glucose homeostasis. In adult female offspring of obese dams, maternal metformin reduced adiposity, improved glucose homeostasis and reduced nonalcoholic fatty liver disease (NAFLD) severity. In adult male offspring of obese dams, metformin had no beneficial effects. In adult offspring of either sex born to lean dams, maternal metformin increased offspring adiposity, impaired glucose homeostasis and worsened NAFLD severity.

Conclusions: Metformin treatment in obese dams partially protects female offspring from the effects of maternal obesity, yet male offspring do not benefit. Offspring of metformin-treated lean dams, of either sex, have more severe metabolic disease. These data urge investigation of mechanisms priming adverse offspring responses to maternal metformin.

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DECLARATION OF AUTHORSHIP

I, ...[please print name]

declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

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I confirm that:

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Definitions and Abbreviations

ACC	Acetyl CoA-carboxylase
ACLY	ATP-citrate lyase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BMR	Basal metabolic rate
C	Chow diet
CCL2	C-C motif chemokine 2
CE	Cholesterol ester
CHD	Coronary heart disease
CPT1	Carnitine palmitoyltransferase 1
CVD	Cardiovascular disease
DAG	Diacylglyceride
DHA	Docosohexaenoic acid
DNL	*De novo* lipogenesis
DOHaD	Developmental Origins of Health and Disease
E#	Embryonic day #
EPA	Eicosopentaenoic acid
FAS	Fatty acid synthase
FBG	Fasting blood glucose
FDA	Federal Drug Administration
FE	Fixed effect
FFA	Free fatty acid

FFM	Fat-free mass
FM	Fat mass
GDM	Gestational diabetes mellitus
H&E	Haemotoxylin and eosin
HF	High-fat
HOMA	Homeostatic model assessment
HSL	Hormone-sensitive lipase
HR	Hazard ratio
iBAT	Interscapular brown adipose tissue
IL	Interleukin
IPGTT	Intraperitoneal glucose tolerance test
LGA	Large-for-gestational age
m	Metformin
MnSOD	Manganese superoxide dismutase
mTOR	Mechanistic target of rapamycin
MUFA	Monounsaturated fatty acid
NAFLD	Nonalcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Nonalcoholic steatohepatitis
NEFA	Nonesterified fatty acid
NHP	Nonhuman primate
NICE	National Institute for Health and Care Excellence
ONPRC	Oregon National Primate Research Center
OPN	Osteopontin

OR	Odds ratio
PCOS	Polycystic ovary syndrome
PUFA	Polyunsaturated fatty acids
RMR	Resting metabolic rate
ROS	Reactive oxygen species
RR	Relative risk
SFA	Saturated fatty acid
TAG	Triglyceride
TIIDM	Type II diabetes mellitus
TLR	Toll-like receptor
TNF	Tumour necrosis factor
WAT	White adipose tissue
WHO	World Health Organisation

Chapter 1: Introduction

1.1 Obesity

Obesity, most commonly defined as a BMI ≥ 30 kg/m^2 and characterised by an excess volume or mass of adipose tissue, is a truly global health concern. In 2008, an estimated 9.8% (205 million) of men and 13.8% (297 million) of women worldwide were obese, rates approximately double those seen in 1980 (4.8% for men and 7.9% for women)[1]. In the UK, the rate rises to an estimated 26% of men and women as of 2010[2]. Despite signs that the rise may be slowing in some specific populations[3], a majority of countries report a continuing increase in overweight and obesity incidence[1]. This increased incidence has resulted in obesity becoming, in the last few decades, a leading cause of morbidity, diminished quality of life and mortality[4].

1.2 Obesity-associated morbidities and mortality

Obesity is a strong risk factor for a number of non-communicable diseases (Figure 1), and is a leading cause of excess global mortality. Two primary mediators of the obesity-related personal and societal healthcare burden are increased risk of type 2 diabetes mellitus (TIIDM)[5] and cardiovascular disease (CVD)[6], with contributions from increased risk of certain cancers[7], and non-fatal disorders such as osteoarthritis[8] and infertility that nevertheless markedly impact on patient quality of life[9]. A recent meta-analysis of obesity-related all-cause mortality studies found individuals with a BMI >35kg/m^2 exhibited increased hazard ratios (HR, 1.29) relative to normal weight individuals (defined as BMI 18.5–25kg/m^2)[10]. In Europe, obesity is estimated to account for between 279,000 and 337,000 excess deaths per year, driven largely by CVD-related (70%) and cancer-related (20%) mortality[11].

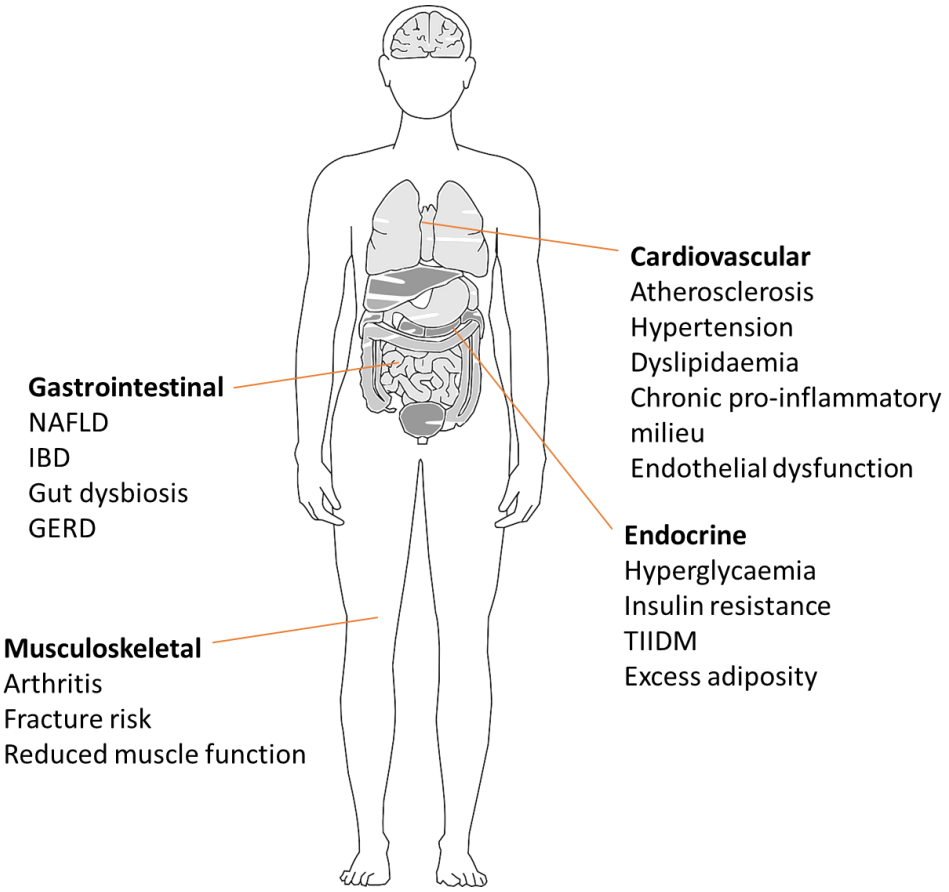

Figure 1 | **Obesity is associated with increased risk of many conditions.** GERD, gastro-oesophageal reflux disease; IBD, inflammatory bowel disease; NAFLD, nonalcoholic fatty liver disease; TIIDM, type II diabetes mellitus. Body illustration is public domain.

1.2.1 Obesity, insulin resistance, and the metabolic syndrome

The metabolic syndrome (MS) is a cluster of cardiometabolic risk factors that predispose individuals to highly elevated risk of TIIDM (fivefold risk increase compared to individuals without MS) and cardiovascular disease (CVD) (twofold risk increase)[12], as well as nonalcoholic fatty liver disease (NAFLD)[13], or cancer[14]. Diagnostic criteria are weighted differently by different defining organisations, yet all agree on the core components of MS: central obesity, glucose intolerance/insulin resistance, hypertension and dyslipidaemia. In the USA, prevalence of the metabolic syndrome in obese individuals (defined by BMI >30kg/m2) is approximately 59.6% and 50% in men and women respectively, corresponding to odds ratios (ORs) of 25.2 and 14 respectively.

Insulin resistance is central to the constellation of metabolic syndrome components. Over-expansion of adipose tissue, particularly of visceral adipose depots (as seen in abdominal obesity), is associated with impaired peripheral insulin function[15]. Resistance to insulin in adipose tissue results in an inability to uptake glucose, exacerbating hyperglycaemia, and increased rates of

lipolysis, resulting in hyperlipidaemia. These excess serum lipids, together with hyperglycaemia and hypertension, constitute a highly atherogenic milieu, and contribute to the increased risk of CVD in patients with the metabolic syndrome[16]. Hyperlipidaemia also contributes to accumulation of lipids in the liver and prevalence of NAFLD in patients with MS, to the extent that NAFLD is now considered the hepatic manifestation of the metabolic syndrome[17]. Around 60% of ectopic fat in the liver is derived from adipose tissue lipolysis[18]. Accumulation of hepatic fat, and particularly free fatty acids (FFAs) or lipotoxic intermediates such as diacylgeride (DAG), is strongly associated with hepatic insulin resistance[19]. This, in turn, contributes to unsuppressed hepatic gluconeogenesis, resulting in hyperglycaemia[20].

1.2.2 Nonalcoholic fatty liver disease

NAFLD is considered the hepatic component of metabolic syndrome, and is now one of the most common causes of chronic liver disease worldwide[21]. Defined as the presence of ectopic fat accumulation in >5% of hepatocytes in the absence of significant alcohol consumption (<20g alcohol per day) or an independent predisposing condition[22], NAFLD represents a spectrum of disease ranging from simple, relatively benign steatosis without hepatocellular damage to non-alcoholic steatohepatitis (NASH) and associated fibrosis. NAFLD progresses to fibrosis in 40–50% of patients, to liver cirrhosis in 15% of those, and full liver failure in 3%[17], representing a significant morbidity, mortality and economic burden worldwide; prevalence of NAFLD in the Western world is estimated at up to 30% of the general population[23], and around two-thirds of the those who are obese[24].

Recently, paired-biopsy studies have suggested that simple steatosis carries significant risk of progression to fibrosis, challenging the view that the condition is benign. In 108 patients with biopsy-proven NAFLD at baseline, 42% had fibrosis progression at a median follow-up time of 6.6 years[25]. This study highlights the importance of interventions to mitigate the development of apparently benign steatosis. Histological scoring of liver biopsy samples remains the gold-standard for diagnosis of NAFLD, despite the intrinsic risks of the procedure[26]. Although circulating levels of liver enzymes are often altered in patients with NAFLD and NASH, plasma concentrations of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are unacceptably poor at diagnosing NAFLD. The entire spectrum of NAFLD can be observed in those with normal ALT and AST levels, values fluctuate within those of healthy individuals on a day-by-day basis, and sensitivity to detect biopsy-proven NASH is poor[27].

NAFLD is strongly associated with presence of overweight or obesity and TIIDM. Although the presence of other components of the metabolic syndrome is associated with increased CVD and

TIIDM risk, NAFLD presence in conjunction with metabolic syndrome exerts a strong, independent risk for both of these diseases[17,28]. This observation reflects the central role of the liver in systemic glucose and lipid homeostasis. Although NAFLD is most frequently seen in individuals who are obese or overweight, some patients develop NAFLD at healthy, lean BMIs. For instance, Feldman *et al.* characterized 187 lean subjects, of whom 55 had NAFLD on ultrasonography and 71 were healthy. Those with NAFLD had higher prevalence of *PNPLA3* gene variants predisposing to NAFLD and impaired glucose tolerance[29]. These data highlight the effect of genetic and epigenetic variants on risk of disease and disease course; NAFLD is not simply a disease of environmental energy excess.

1.2.2.1 Pathogenesis of NAFLD

Development of steatosis is the result of dysregulation of the coordinated processes regulating hepatic lipid homeostasis (Figure 2). These processes comprise: uptake of dietary fatty acids; uptake of lipolysed fats from peripheral sources, predominantly adipose tissue; synthesis of fatty acids from acetyl-CoA in a process known as *de novo* lipogenesis (DNL); export of fatty acids via VLDL; and fatty acid oxidation[30].

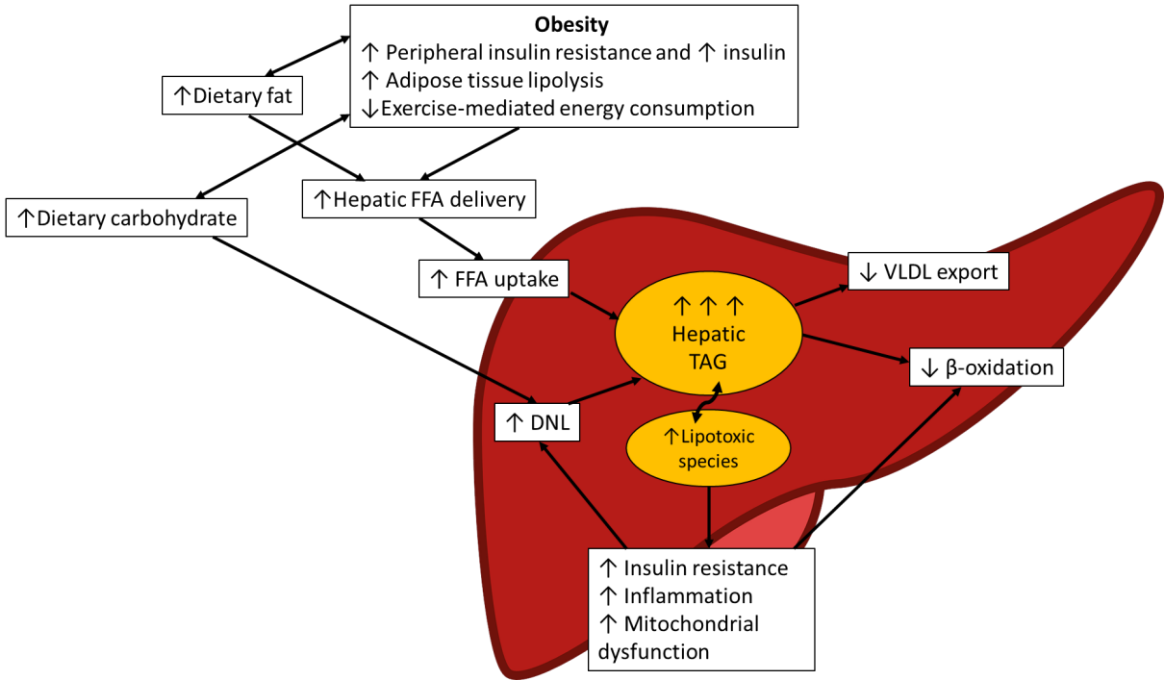

Figure 2 | **Mechanisms promoting development of NAFLD.** DNL, *de novo* lipogenesis; FFA, free fatty acid; TAG, triglyceride; VLDL, very low density lipoprotein. Liver illustration is public domain.

1.2.2.2 Contribution of peripheral adipose tissue

Delivery of FFA from peripheral adipose tissue is important to the initial development of NAFLD, given approximately 59% of fat present in hepatocytes in obese patients with NAFLD originates

from adipose tissue, compared to approximately 26% from DNL and 15% from diet[18]. Insulin resistance in peripheral tissues, as often seen in obese individuals, fails to supress hormone-sensitive lipase (HSL) activity, resulting in increased release of FFA from adipose tissue into circulation. Lipid flux away from adipose tissue towards leads to increased hepatic lipid uptake via the hepatic portal vein; in the absence of increased oxidation or export of lipid, accumulated hepatic lipid will form steatotic vesicles in hepatocytes[17]. In a striking illustration of the role of adipose tissue in NAFLD development, patients with lipodystrophy suffer from severe accumulation of hepatic lipids as a result of their inability to store fat peripherally. In one study, 86% of patients with lipodystrophy had biopsy-proven NASH at baseline, despite having low BMI ($<25 kg/m^2$)[31].

1.2.2.3 Hepatic insulin resistance and NAFLD

In addition to peripheral insulin resistance resulting from obesity promoting NAFLD, presence of fatty liver is independently associated with impaired hepatic insulin sensitivity[32]. Moreover, ectopic fat accumulation in the later stages of NAFLD is associated with a worsening of insulin resistance, suggesting an additive effect of NAFLD presence[33]. Results from animal models and human studies have focused on two primary mechanisms for NAFLD-associated insulin resistance: disruption of hepatic insulin signalling caused by DAG-mediated activation of protein kinase Cε[34]; and inhibition of AKT activation by accumulation of ceramide as a result of inflammation-mediation reductions in insulin signalling[35]. AKT is a key mediator of the effects of insulin signalling, activating downstream pathways promoting cell survival, glucose uptake and DNL, amongst others[36,37]. Recent work has suggested DAG accumulation, an intermediate in increased hepatic DNL, to be a more probable contributor to hepatic insulin resistance than ceramides, due to absence of increased ceramide levels in patients with NAFLD[38]. Crucially, hepatic insulin resistance results in a paradoxical failure of insulin to suppress hepatic gluconeogenesis while still stimulating lipogenesis. This probably occurs because of a bifurcation in the insulin and mechanistic target of rapamycin (mTOR) signalling pathways. Thus hepatic insulin resistance drives DNL, promoting a vicious cycle of hepatic triglyceride (TAG) accumulation[39] and excessive hepatic gluconeogenesis, contributing to the pathology of metabolic syndrome[17].

1.2.2.4 Progression to NASH

Not all patients with NAFLD progress to NASH, raising the question of why ectopic fat deposition leads to steatohepatitis in some individuals and not others[40]. The disposition towards NAFLD progression is probably the result of a number of integrated genetic, environmental and epigenetic factors – these drivers constitute a “multi-hit” model that has evolved from a “two-hit” hypothesis, which comprised an initial accumulation of triglyceride in hepatocytes followed by a

secondary inflammatory insult[41]. A number of interconnected factors have been explored, focusing on lipotoxicity, oxidative stress, mitochondrial dysfunction and mediators of the inflammatory response.

Fatty acids, whether consumed in the diet or derived from peripheral adipose tissue lipolysis, differ in their physiological actions in the liver. Whereas n-3 polyunsaturated fatty acids (PUFAs, such as docosohexaenoic acid (DHA) or eicosapentaenoic acid (EPA)), are broadly anti-inflammatory[42], n-6 PUFAs (such as linoleic acid) are pro-inflammatory, being precursors in the synthesis of prostaglandins and leukotrienes, amongst other molecules[43]. Studies of the hepatic n-6:n-3 PUFA ratio in patients with or without NASH have shown that an increasing level of n-6 PUFAs relative to n-3 PUFAS is associated with increased disease severity[44]. Consequently, it is not surprising that increasing hepatic n-3 PUFA levels in a rodent model of NAFLD decreases hepatic inflammation[45]. Although studies have shown that n-3 PUFA supplementation in large doses can reduce hepatic steatosis in patients with NAFLD[46], there is no evidence yet for the ability of dietary n-3 PUFAs to reverse hepatic inflammation in humans.

Although hepatic TAG levels are associated with metabolic derangements, there is evidence to suggest that accumulation of TAG might be a protective adaptation to protect the liver from the effects of lipotoxic TAG metabolites[47]. In a mouse model of NAFLD, blocking TAG synthesis in obese mice by inhibiting diglyceride acyltransferase, the enzyme catalysing the final step in TAG synthesis in hepatocytes, dramatically exacerbates steatohepatitis, as a result of increased hepatic levels of FFA[48]. Conversely, overexpressing DGAT2, and thus increasing TAG synthesis capacity, increases levels of steatosis but does not impair normal systemic or hepatic glucose metabolism in mice[49].

As a result of data showing that hepatic steatosis is readily uncoupled from derangement of hepatic glucose homeostasis and development of NASH, additional mechanisms have been sought to explain the metabolic dysfunction and inflammation observed in patients with NAFLD and NASH. Individuals who progress to NASH show increased hepatocyte oxidative stress, as a result of both increased lipid peroxidation necessitated by FFA overload of the mitochondrial β-oxidation pathway[50] and increased expression of the microsomal fatty acid oxidising enzyme CYP2E1[51]. Overexpression of CYP2E1 in murine models results in impaired hepatic insulin signalling and increased oxidative stress and TAGaccumulation[52].

As the primary site of reactive oxygen species (ROS) generation, mitochondria are especially vulnerable to increases in oxidative stress. Defects in mitochondrial function have been reported in many clinical and animal model studies of NASH[53-55], with suggestions that mitochondrial dysfunction may precede NASH development in some cases[54]. Decreased ATP production in spite

of increased ROS production has been reported in models of NASH[56]; UCP2, a mitochondrial uncoupling protein typically expressed to reduce ROS production at the expense of cellular ATP generation, was found upregulated in animal models of NASH[57]. NASH patients also show alterations to mitochondrial morphology[58] and reduced mitochondrial DNA (mtDNA) integrity[59]. Critically, patients with obesity, with or without NAFL but without NASH retain the ability to upregulate hepatic mitochondrial respiration, whereas obese patients with NASH, despite having greater mitochondrial mass, have lower maximal mitochondrial respiration rates[60]. These obese NASH patients also showed greater mitochondrial proton leakage and increased hepatic insulin resistance and inflammation compared with patients without NASH.

As well as driving hepatic insulin resistance, hepatic accumulation of lipotoxic moieties can lead to the activation of inflammatory pathways or induce hepatocyte apoptosis. For instance, increased levels of the saturated FFAs stearate or palmitate induce lipoapoptosis, whereas unsaturated FFAs do not[61]. Mice with deletion of stearoyl CoA desaturase-1 (*Scd1*), an enzyme that catalyzes the synthesis of monounsaturated FFAs from saturated FFAs and ultimately the synthesis of TAG, accumulate hepatotoxic saturated FFAs; in spite of decreased hepatic TAG levels, these mice show dramatic hepatic inflammation, lipoapoptosis and fibrosis[62]. High mitochondrial levels of free cholesterol, seen in mouse models of NAFLD, sensitize hepatocytes to the pro-inflammatory actions of TNF (plasma levels of which are increased in obese patients) and the development of NASH[63]. Free cholesterol accumulation has also been linked to hepatic inflammasome activation and fibrosis in mice[64], and human patients with NASH, but not simple steatosis, have increased hepatic levels of free cholesterol[65].

1.2.2.5 Early-life priming of NAFLD and NASH

Recently, the uterine environment during fetal development has been linked to risk of subsequent development of NAFLD and NASH in offspring[66]. The mechanisms and studies underpinning this association are discussed in detail later in this chapter, but core supporting principles are outlined below.

In humans, exposure to maternal diabetes during pregnancy is independently associated (adjusted OR 6.74) with risk of NAFLD (assessed by ultrasound scan) in offspring at mean 17.8 years; this association was not mediated by offspring birth weight or adiposity, suggesting persistent, liver-specific perturbations in offspring[67]. Moreover, fetuses or neonates born to mothers with diabetes already display hepatic steatosis[68,69]. Mice exposed to maternal obesity during development and challenged with a high-fat (HF) diet post weaning showed, at 30 weeks of age, increased hepatic TAG accumulation, leukocyte infiltration, markers of hepatic fibrosis and inflammation, and histologically-defined NASH, compared with animals only exposed to post-

weaning HF-diet. This finding was accompanied by reduced mitochondrial electron transport chain (ETC) activity in animals only exposed to HF-diet *in utero*, an indication that mitochondria are at risk from adverse developmental conditions and that this effect is persistent [70]. Impaired ETC activity can lead to hepatic lipid accumulation by suppressing the capacity to oxidise fatty acids via β-oxidation[71]. Decreases in offspring hepatocyte mitochondrial copy number after exposure to maternal obesity and/or HF-diet have also been shown in animals that have increased risk of developing NASH[72,73].

In addition to impacts on mitochondrial function and number, a maternal HF-diet during pregnancy results in fetal fatty liver and hepatic and systemic inflammation and oxidative stress in a non-human primate (NHP) model[74]. Thus, developmental exposure to HF overnutrition resulting in fatty liver is not a benign condition, and might exacerbate progression to NASH if the fetus is challenged with an obesogenic diet postnatally.

Taken together, there is considerable scope for considering maternal high-fat and/or obesity-mediated developmental 'priming' of NAFLD and NASH susceptibility a 'first hit' in the pathogenesis of the disease [75]. The term priming is considered more appropriate than 'programming', as the former does not imply a wholly deterministic process [76]. Although the terms are used interchangeably throughout this thesis, this point should be born in mind, particularly when one considers that the deleterious effects of maternal obesity during pregnancy may not manifest in animal models unless a postnatal HF diet is given, or in humans unless an obesogenic environment is encountered postnatally.

1.2.2.6 Obesity and cardiovascular disease

Obesity is strongly implicated in the risk of coronary heart disease (CHD) (relative risk (RR) 1.81[6]), the primary cause of early mortality in the Western world[77]. This association remains after adjustment for blood pressure and cholesterol levels (RR 1.49), suggesting an effect of obesity on CHD risk independent of those variables. Obesity is additionally strongly associated with increased risk of heart failure[78], myocardial infarction[79], and hypertension[80]. These associations remain significant yet are heavily attenuated by complete adjustment for independent cardiovascular risk factors, including facets of MS.

1.2.2.7 Additional obesity-associated disorders

Renehan and colleagues conducted a large meta-analysis of studies investigating links between BMI and 20 different cancer types. They identified significantly increased risk of oesophageal adenocarcinoma (RR 1.52), thyroid (RR 1.33), and colon (RR 1.24) cancers in men with a 5kg/m^2 increase in BMI. In women, a 5 kg/m^2 increase in BMI increased risk of endometrial (RR 1.59),

gallbladder (RR 1.59), oesophageal adenocarcinoma (RR 1.51), and renal (RR 1.34) cancers[7]. Prognosis in many common cancers is significantly worsened by the presence of obesity (including breast[81], colon[82] and prostate[83] cancers).

Obesity is also associated with a number of non-fatal, debilitating or burdensome conditions that impact markedly on quality of life. These include impacts on respiratory function (including decreased lung volume[84] and risk of obstructive sleep apnoea[85]), the skeletal-muscular system (such as increased incidence of osteoarthritis in both weight-bearing and nonweight-bearing joints[8]) and the reproductive organs (including risk of polycystic ovary syndrome (PCOS)[86], and reduced male[87] and female[88] fertility).

1.2.3 Pathogenesis of obesity

At an individual level, chronic weight gain leading to eventual obesity necessitates a chronic net intake of energy. In its simplest terms, net energy excess occurs as a result of energy over-consumption, with a concomitant lack of increase in energy expenditure over time, both factors enabled by the broad socioeconomic changes in the recent past[89]. When energy taken in from the diet exceeds energy expended, excess energy is stored. Chronically, this stored energy manifests as increased storage of TAGs in adipose tissue and ectopically within other organs.

The mechanisms responsible for net energy excess at both a population and an individual level are diverse and often controversial. In general, social, economic and cultural has driven the rise of obesity at a population level[89]. The World Health Organisation (WHO) has identified increased cheap food availability and aggressive marketing as drivers of consumption, in addition to the reduced energy expenditure inherent with increasing mechanisation and sedentarianism. Global increases in high-energy density food availability[90-92] and total energy consumption[93,94], and decreases in physical activity[95], have occurred in the past few decades and have been implicated in the growing obesity trend. In sum, these changes constitute an obesogenic environment that mismatches substantially from the environment present during human evolution[96].

1.2.4 Parental obesity and offspring metabolism

The overall increase of obesity prevalence has led to a doubling of pregnancies complicated by the condition (a prevalence of 15.6% in first trimester pregnancies in the UK in 2007, up from 7.6% in 1989[97]). Maternal obesity is associated with increased health risks to both the mother and child in the short-term (during gestation and perinatally) and long-term (during the postnatal development of the offspring and their adult life)[98]. Although paternal obesity has also been

linked to adverse effects on offspring metabolic health[99], a discussion of this field is outside the scope of this thesis.

1.2.4.1 Short-term effects of maternal obesity on the mother

The conditions associated with obese pregnancy mirror those seen in individuals with metabolic syndrome, and are likely driven by the decreased insulin sensitivity inherent during pregnancy, coupled with likelihood of existing perturbations to metabolic system incurred by obesity[100].

Obese women exhibit an increased risk of gestational hypertension and preeclampsia, with risk increasing with obesity severity and presence of preconception hypertension[101]. A large meta-analysis of the role of pregravid BMI on incidence of gestational diabetes (GDM) reported ORs of 1.97, 3.01 and 5.55 for overweight, obese and severely obese women, respectively, compared to healthy weight controls[102]. This dramatically increased risk of GDM is perhaps unsurprising given the pregravid insulin insensitivity associated with increasing overweight and obesity in women[103]. In the landmark Hyperglycaemia and Adverse Pregnancy Outcomes (HAPO) study, 25,505 pregnant women from multiple countries underwent a glucose tolerance test between weeks 24-32 of pregnancy before details on birth outcomes were recorded[104]. The researchers found that there were strong, continuous relationships between maternal blood glucose concentrations below pathological levels and birth weight and cord-blood C-peptide levels. These results highlight the importance of maintaining appropriate blood glucose control, even in the absence of overt diabetes, and demonstrate the programming effects of moderately elevated maternal blood glucose.

Insulin resistance also drives disruption of lipid metabolism, which is altered during the course of normal pregnancy. Increases in circulating FFAs, driven by adipose tissue lipolysis that is ordinarily suppressed by the action of insulin, are larger in women with GDM compared to insulin sensitive women[105].

Pregnancy is associated with an altered inflammatory state, required to ensure appropriate implantation and trophoblast invasion and to maintain placental function [106]. Obesity also alters the inflammatory state, increasing circulating levels of pro-inflammatory markers. A number of studies assessing longitudinal changes in maternal plasma cytokine levels have shown that levels of C-reactive protein (CRP) and IL-6 are increased during obese pregnancy relative to lean pregnancy, whereas levels of TNF do not seem to be substantially increased[106].

1.2.4.2 Short-term effects of maternal obesity on the fetus and neonate

Infants of obese women are more frequently large-for-gestational-age (LGA) than those of control women (16.8% vs 10.5% of deliveries, respectively)[107]. There are suggestions that fetal overgrowth may be partially mediated by the dysregulation of FFA supply to the fetus, as evidenced by strong correlations between maternal TAGs and fetal growth, in addition to altered placental transport of FFA in GDM pregnancies[108] and altered regulation of fatty acid metabolism genes[109]. The HAPO study also demonstrated strong, linear association between maternal blood glucose levels and neonate body weight, suggesting a role for maternal glucose supply in regulating fetal growth[104].

Maternal obesity conveys nearly double the risk of stillbirth compared to mothers of a normal weight[110] and increases risk of early neonatal death[111]. Risk of a range of congenital birth defects is increased in offspring of obese mothers compared to normal weight mothers, including neural tube defects (OR 1.87), cardiac defects (OR 1.30), and cleft lip/palate (OR 1.20)[112].

Catalano *et al.* demonstrated that fetuses born to obese mothers had greater insulin resistance (as assessed by the homeostatic model assessment (HOMA)), degree of adiposity and pro-inflammatory IL-6 levels in cord blood, compared with fetuses from lean women[113]. However, other studies have failed to show increased cord blood cytokines in offspring from obese mothers, despite increased maternal plasma and placental inflammatory markers[114]. Noninvasive imaging of neonates born to mothers with diabetes has shown increased levels of intrahepatic fat that correlate with maternal BMI, suggesting priming of the early stages of NAFLD in offspring[68]. Histopathology demonstrated increased steatosis in stillborn fetuses from mothers with diabetes[69].

1.2.4.3 Long-term effects of maternal obesity on offspring in humans

The consequences of maternal obesity are now believed to extend beyond the immediate postnatal period. Barker and colleagues first characterised an increase of CHD in areas with high infant mortality 40 years previously[115]; this finding was supplemented by the demonstration of strong links between low birth weight and subsequent risk of death from CHD[116] and incidence of TIIDM and MS[117]. This led to the formation of the Developmental Origins of Health and Disease (DOHaD) hypothesis, and the postulation of the existence of a 'thrifty phenotype' in the fetus in response to inadequate maternal nutrition. This developmentally programmed conservation of resources by the fetus, in anticipation of being born into a world of resource deprivation, results in severe imbalance between expecting and encountered nutritional availability when exposed to typical western diets in later life[118].

Evidence of the converse, an association between high birth weight and risk of offspring metabolic disease, was provided shortly thereafter[119], suggesting the curve of birth weight and risk was in fact U-shaped[120]. Such an association is especially relevant in the West, given the increasing proportion of pregnancies affected by maternal obesity. A series of elegant sibship studies demonstrated a dramatically increased risk of TIIDM and obesity in offspring exposed to a diabetic intrauterine environment, with no relationship with paternal diabetes[121-123]. Exposure to maternal obesity in the absence of GDM or TIIDM and subsequent macrosomia is predictive of offspring bodyweight at 1 year, which in turn is highly predictive of adiposity at 5-8 years[124]. Maternal obesity, independent of GDM or TIIDM, was found to increase offspring risk of MS at 11 years of age (HR 1.81)[125]. Similarly, maternal obesity in the first trimester of pregnancy was significantly associated with increased risk of childhood obesity at 2, 3 and 4 years of age (RR 2.0, 2.3 and 2.3 respectively)[126].

The independent contribution of maternal obesity and its associated metabolic disturbances was demonstrated in studies of large maternal weight loss between multiple pregnancies[127,128]. Children born after maternal bariatric surgery had reduced prevalence of macrosomia, and improved metabolic profiles and a 3-fold reduction of severe obesity prevalence when compared to their siblings born before bariatric surgery, at follow-up at 26 years of age[128]. In a similar study, the DNA methylome (the genomic landscape of methyl groups attached to DNA CpG dinucleotides, which epigenetically regulate gene function) and metabolic profile of siblings born before or after maternal bariatric surgery for obesity was assessed at mean 12 years follow-up[129]. Offspring born after bariatric surgery had less body fat, lower fasting insulin and reduced insulin resistance and had lower blood pressure compared with siblings born before bariatric surgery; additionally, offspring born after maternal surgery had markedly different methylation of genes associated with glucose metabolism and homeostasis and inflammation.

1.2.5 Animal models of developmental priming

Animal models have proved invaluable for studying the influence of maternal metabolic health on their offspring for a number of reasons. Primarily, animal models allow the probing of maternal and fetal tissue at sensitive time-points, using procedures (such as liver biopsy) that are considered too dangerous and ethically unacceptable to perform on otherwise healthy individuals, particularly babies or infants[130]. In addition, animal models allow exact control of experimental conditions, including timing and composition of obesogenic diets, timing of conception, and minimization of confounding influences that are inescapable in human studies. Studies are also far quicker to perform in animals with short gestational periods, such as the mouse or rat, where gestation is ~3 weeks. Despite these advantages, data from animal models

should be interpreted with a degree caution on the basis of physiological differences between experimental organisms and humans. For instance, the typical litter size of 8–10 pups in rodent pregnancy is at odds with the typical singleton or twin pregnancy in humans. Relevant to metabolic disease, a further example of comparative differences between humans and model species is the possession by rodents of a relatively large mass of brown adipose tissue (BAT) in contrast to the small amounts possessed by adult humans[131]. BAT in rodents is crucial for nonshivering thermogenesis on exposure to cold; thermoneutrality in rodents is higher than in humans, at around 30˚c, greater than the 22˚c commonly found in animal housing units. Thus, the relative contributions of metabolic pathways to whole-body energy expenditure could influence the metabolic phenotype of the animal in a manner not consistent with the response in humans[132].

Supporting data from human studies, animal models have made it clear that a large range of factors dependent on the maternal metabolic status are capable of conveying risk to the offspring. These factors include: the effect of maternal obesity *per se* and associated physiological disturbances; the independent impact of an obesogenic or overnutrition diet during pregnancy; the macro- and micronutrient make-up of that diet; and the timing of the dietary or obesity insult in relation to conception and development of the fetus. The impact of a secondary obesogenic insult directly to the offspring after weaning is also often assessed, modelling the widespread overconsumption of energy in humans.

1.2.6 Animal models and alteration of fetal physiology

An investigation of fetal responses to maternal obesity or obesogenic feeding is critical to understanding the mechanisms driving developmental priming. Despite this, there exists a relative paucity of studies reporting impacts on fetal physiology.

A recent series of comprehensive studies conducted at the Oregon National Primate Research Centre (ONPRC) investigated the impact on third-trimester fetal offspring of maternal obesity and HF-feeding in a Japanese macaque model, characterising a range of early impacts on fetal physiology. In the fetal liver, of particular interest due to its central role in glucose homeostasis, they demonstrated a threefold increase in hepatic TAGs in offspring of HF-mothers, along with increased gluconeogenic gene expression and oxidative stress, all factors associated with development of NAFLD in adult animal models[74]. They also showed increased hepatic apoptosis yet no increase in hepatic pro-inflammatory factors[133]. Interestingly, diet reversal during pregnancy alleviated much of the adverse fetal phenotype, suggesting independent impacts of maternal diet and obesity. Additional changes were seen in hepatic chromatin structure and heat-

shock protein expression[134], and hepatic peripheral circadian clock gene expression[135] and *Sirt1* expression and activity[136]. Hypothalamic feeding circuitry was also altered in this model. Abnormalities were seen in hypothalamic expression of components of the anorexigenic melanocortin system and the development of central appetite regulatory circuits[137]. These changes could be expected alter appetite and control of energy intake.

It remains to be seen whether the specific fetal changes observed in the ONPRC NHP model have the potential to persist, and are causally related to the altered adult phenotype in animals exposed to high fat *in utero*. However, the evidence is suggestive of a strong effect on fetal development in systems shown to be disrupted in adulthood by independent high-fat/obesity exposure.

Despite being used widely in maternal undernutrition studies, ovine models of maternal obesity are less common. Models of maternal overnutrition prior to conception and through gestation have characterised a number of effects on fetal physiology. These include reductions in the activity of the AMPK system and insulin signalling in skeletal muscle[138], key pathways in the maintenance of glucose homeostasis, as well as impaired skeletal development in fetuses from overnourished mothers[139]. Critically, given the propensity of *in utero* HF-exposed offspring to develop cardiovascular dysfunction in later life, Wang and colleagues detailed increased stress markers, impaired cardiac insulin signalling and impaired cardiac output in HF-exposed fetuses[140]. Fetal adipose tissue in these fetuses also showed substantial changes, with increased protein expression of fatty acid translocase, fatty acid transport proteins 1 and 4, and adipocyte hypertrophy[141], suggesting early-life priming of increased adiposity.

Rodent models of fetal exposure to HF and obesity during development have shown similar detrimental effects of maternal overnutrition on numerous organs. In the brain, these include altered hippocampal development[142] and dysregulated hypothalamic expression of appetite and insulin signalling pathway genes[143]. In adipose tissue, increased expression of markers of inflammation and decreased *Glut4* mRNA were seen[144]. Skeletal muscle is also altered by the effects of maternal obesity. Tong et al. showed downregulated levels of myogenic factors, and increased expression of the adipogenic protein PPARγ, in the skeletal muscle of fetal mouse pups exposed to maternal obesity *in utero*[145].

As in NHP models, the liver is an attractive investigative target, and rodent models display many similar alterations to primates to an HF insult. El-Sayyad and colleagues showed hepatocyte DNA damage in HF-exposed fetuses, and elevated markers of hepatic apoptosis. This apparent impact of HF-exposure on liver development has been corroborated by other studies[146,147]. Heerwagen *et*

al. also showed increased liver TAG accumulation in conjunction with increased expression of placental lipid transporter[148].

Thus, data from diverse animal models support the notion that maternal obesity affects numerous fetal developmental pathways relevant to long-term cardiometabolic disease risk[130].

1.2.7 Independent contributions of diet and obesity

Animal models utilising diet reversal prior to conception or obesogenic diet feeding limited to critical development windows have attempted to delineate the independent contributions of maternal obesity and an obesogenic diet to developmental priming. McCurdy *et al.*[74] reported partial rescue of fetal metabolic health by replacing HF diet with C diet during pregnancy in obese non-human primate (NHP) females. Similar partial rescue was reported in mice[149]. Feeding of an HF diet to female mice solely during pregnancy and lactation was also found to impair offspring metabolic parameters to a similar degree seen in dams fed HF diet for 5 weeks prior to conception[150]. Supplementing these findings, White *et al.* employed a calorie-controlled feeding protocol in rats and found that consumption of HF diet by non-obese dams was not responsible for developmental priming, providing calorific intake was equal to that of chow (C) fed dams[151]. This suggests, in non-obese animals, an impact on developmental priming dependent on calorific intake rather than macronutrient composition. While the use of non-standardised animal models precludes definitive answers on individual contributions of maternal diet and obesity, each clearly has a role in priming offspring metabolic dysfunction[152]. Thus, clinical interventions are required that target both of these contributory factors to achieve maximum protection of offspring[153].

1.2.8 Importance of developmental windows

It is also evident that the timing of the insult to different developmental windows, from preconception to lactation, is important. Oocytes isolated from female mice fed an obesogenic diet for 6 weeks showed increased generation of reactive oxygen species and depletion of antioxidants, in conjunction with increased mitochondrial biogenesis and impaired zygote survival[154]. Further studies have demonstrated reduced oocyte quality in female mice after obesogenic diet feeding for several weeks[155,156], and showed significantly increased oocyte lipid content and mitochondrial stress markers[157]. Culture of mouse embryos with high concentrations of palmitic acid (PA) and subsequent implantation in C-fed surrogate dams yielded pups growth restricted at birth, which then overtook control pups after catch-up growth[158]. A lack of studies following up HF-diet exposed embryos into adulthood precludes assessment of the long-term impact of very early development exposure to an obesity milieu, yet the catch-up growth

demonstrated by growth-restricted pups after palmitic acid exposure is a marker in humans and rodents of risk of adult metabolic diseases[159].

A large number of animal models of developmental priming utilise obesogenic feeding from pre-conception through development to mimic conditions seen in a clinical setting. Typically mothers are rendered obese prior to conception, with *ad libitum* access to an obesogenic diet for around 4-8 weeks in rodents[130] or several years in a non-human primate model[74]. This model does not allow the separation of the individual contributions of early and late exposures, but does effectively recapitulate the majority of human fetal exposures, wherein mothers do not suddenly and drastically alter diets upon conception, and is therefore an attractive model to assess the potential efficacy of interventions.

1.2.9 Acute impacts of maternal obesity on placental and fetal growth

A number of studies have demonstrated impacts of maternal obesogenic diet exposure on placental function and fetal growth, albeit with conflicting findings. Jansson and colleagues reported large increases in the expression of key placental nutrient transporters, including the glucose transporter GLUT1 and the amino acid transporter SNAT2 in placentas from HF-fed, yet non-obese mice. This was accompanied by a 43% increase in fetal weights at embryonic day 18.5 (E18.5)[160]. In a report from the same group using a different mouse model (obese dams fed an obesogenic diet), maternal obesity was associated with increased fetal weight and increased expression of placental transport proteins, including GLUT1, SNAT2 and LAT1, at E18.5[161].

By contrast, recent work demonstrated increased glucose and neutral amino acid transfer in placentas of mice fed HF-diet from mating at E16, with decreased fetal and placental weight, yet both nutrient transfer and weights had normalised by E19[162]. In a rat model of HF-feeding and obese pregnancy reduced placental junctional zone weight and yielded fetal growth restriction at E21 [163]. In yet another model, male fetuses were growth restricted at E18 after exposure to a maternal HF diet and moderate maternal obesity; female fetuses were unaffected, yet placentas from both male and females fetuses were smaller than placentas from control dams[164].

Recent work investigating the role of mTOR signalling in placental nutrient transport, suggested in humans as a mediator of fetal-maternal nutrient transfer regulation [165], found an unexpected decrease in mTOR activity in placentas from HF-fed, obese mice, with no impact on fetal or placental growth[166]. However, in humans, mTOR signalling has been found to be upregulated in placentas from obese pregnancy[167]. Birthweight was significantly correlated to increased placental mTOR and insulin signalling pathways and the upregulation of SNAT2. Thus, in humans at least,

maternal obesity seems to prime increased placental nutrient transport, which might be responsible for increased fetal growth.

Placental inflammation as a result of maternal obesity has been advanced as a potential mechanism influencing fetal growth via alterations to placental structure and efficiency, and induction of a fetal inflammatory response[168]. In an ovine model of maternal obesity, placentas from obese mothers showed higher expression of inflammatory markers toll-like receptor 4 (TLR4), toll-like receptor 2 (TLR2) and the cytokines tumour necrosis factor (TNF), and IL-6, IL-8 and IL-18[169]. This may be linked directly to raised circulating FFA concentrations seen in obesity, which are capable of activating TLR4 directly, driving transcription of inflammatory cytokines[170]. Importantly, increased placental inflammation is also a feature of obese human pregnancy. Challier *et al.* found markedly increased levels of placental CD68+ and CD14+ macrophages, and increased placental IL1, TNF and IL-6 levels, in obese pregnancy compared with lean pregnancy[171].

There are clear discrepancies between studies in fetal growth as a result of maternal overnutrition and/or obesity. These differences have been ascribed to animal strain, dietary components and maternal phenotype variability with no definitive answer[130], yet it is clear that maternal obesity and overnutrition is able to disrupt fetal growth trajectories and placental function.

1.3 Impacts of maternal overnutrition on adult offspring physiology and disease risk

Exposure to maternal over-nutrition and/or maternal obesity is, as discussed earlier, associated with increased disease risk in adulthood, especially when accompanied by postnatal overnutrition. As seen in fetal studies, developmental priming affects the entire organism, either directly or indirectly.

Exposure to a high-fat diet, as in humans, results in increased risk of obesity and overweight in the offspring. Animal models have demonstrated increased adiposity and body fat percentage in adult offspring, independent from yet exacerbated by postnatal diet, in line with human data[70,172,173].

A number of groups have described impaired cardiovascular function in adult offspring of obese, overnourished mothers. In rodents, this includes hypertension independent of postnatal diet[172,174], which is present early in life prior to the development of overt metabolic disturbances, suggesting a specific *in utero* programming effect[175]. This early-life impact on juvenile cardiovascular function is also seen in a NHP model[176]. Interestingly, treatment of hypercholesterolaemic pregnant mice with a statin during the pre-mating HF feeding phase resulted in protective effects on dams (reduced total plasma cholesterol and systolic blood

pressure (SBP)) and their adult offspring (reduced body weight and protection against hypertension), suggesting a hypertensive mechanism at least partly dependent on adversely altered maternal lipid metabolism[177]. Endothelial dysfunction in offspring was also seen in a number of studies in rodents[172,178,179], and in the ONPRC NHP model[180].

Offspring of obese HF mothers also display liver dysfunction, both independent of postnatal diet and exacerbated by it. Bruce *et al.* showed fatty liver in offspring exposed to maternal HF alone, and NASH in animals exposed to both *in utero* and postnatal overnutrition[70]. These animals showed up-regulation of lipogenic genes, impaired mitochondrial function and reduced fatty acid oxidation, a finding seen in other models[72,181,182]. Such a pattern of hepatic metabolic dysfunction primed by exposure to maternal over-nutrition has been considered to be the first of the multiple hits implicated in NAFLD progression to NASH[70]. Certainly, the findings from studies of fetal livers exposed to maternal overnutrition in animal models discussed above suggest a strong developmental role in increasing NAFLD and NASH risk, making modulation of developmental priming of potential key important in reducing offspring NAFLD risk[66]. However, long-term longitudinal studies assessing early-life NALFD-associated changes in the liver (such as neonatal steatosis) with adult offspring NAFLD incidence and disease severity are lacking.

Adult offspring adipose tissue is also susceptible to maternal developmental priming. A recent mouse study showed altered adipokine secretion in offspring of HF-fed mothers, including elevated leptin and lowered adiponectin levels[183]. However, whether these changes are as a direct result of maternal priming or a consequence of adipose tissue expansion resulting from altered offspring metabolic phenotype is unclear. Umekawa *et al*. showed that offspring of HF fed mothers had, independent of postnatal diet, increased mesenteric adipose tissue, and increased infiltration of pro-inflammatory macrophages and decreased serum adiponectin[184].

Differential regulation of feeding via changes to appetite and reward systems has been described in a number of well-characterised animal models – animals exposed to maternal HF-diet or obesity are typically hyperphagic as adults. Mice from dams fed a high-energy diet showed increased preference for high-energy foods in adulthood, combined with increased expression of dopamine-synthesis related genes, a finding linked in other studies to increased food intake[185]. This finding was replicated by another group in a similar rodent model[186]. In yet another study, offspring of obese rats demonstrated significant leptin resistance by 30 days of age, an effect that persisted to at least 90 days of age and was accompanied by hyperphagia, hyperleptinaemia and obesity[187].

1.4 Interventions to alleviate developmental priming

In light of the wealth of evidence supporting the detrimental impact of maternal obesity and HF-diet feeding on offspring health in adulthood, attention is turning to suitable interventions to provide protection to both mother and child during pregnancies impacted by overnutrition[153]. Lifestyle interventions (involving diet and exercise programs) show limited efficacy in humans. A recent review of maternal lifestyle intervention studies suggested lifestyle intervention, could yield a modest (2.21kg) reduction in gestational weight gain. However, the same review highlighted the lack of impact on antenatal measures, including large-for-gestational-age, birth weight and macrosomia, and no effect on maternal GDM incidence[188]. The UPBEAT trial, a randomized controlled multicentre trial of an intensive behavioural intervention for obesity in 1,555 pregnant women with a BMI >30kg/m^2, found no difference in the primary outcomes (GDM and LGA incidence) in women receiving the intervention or standard care[189]. However, the intervention did improve maternal diet and levels of physical activity, and modestly decreased gestational weight gain by 0.55 kg. These data point to the limited effectiveness of lifestyle interventions, which are only typically initiated at 10 to 12 weeks gestation, at the first antenatal appointment. Catalano and deMouzon have suggested that this time point is too late to initiate an effective intervention, given data from human studies and animal models showing marked and persistent alterations in response to maternal obesity from conception, for instance priming placental function during the first trimester[190]. Pharmaceutical and nutritional interventions offer an alternative therapy with potentially greater compliance and effectiveness and the opportunity to use such interventions prophylactically, yet research into the impact of such interventions on offspring outcomes is limited. There is undoubtedly concern that pharmacological or nutritional interventions might be teratogenic, limiting investigation of candidate therapeutics to those with proven safety in pregnancy.

1.4.1 Nutritional Interventions

Particularly attractive are interventions capable of influencing both maternal and placental inflammation, factors implicated in adverse obesity-mediated metabolic priming. This has been attempted in animal models in several ways.

A recent murine study investigated the impact of increasing the maternal ratio of anti-inflammatory n-3 PUFA to pro-inflammatory n-6 PUFA in pregnant dams on their offspring[148]. This was achieved with a transgenic mouse strain capable of endogenously converting n-6 to n-3. Maternal systemic and placental inflammation induced by HF-diet was significantly reduced by an increased n-3:n-6 ratio. A range of fetal and placental parameters, including fetal:placental

weight, TAG accumulation, and placental lipoprotein lipase activity were restored to control levels in HF mothers by raising the maternal n-3:n-6 ratio. Adult male offspring of high n-3:n-6 mothers had improved metabolic profiles, including reduced liver TAGs, improved insulin sensitivity and glucose tolerance, reduced adipose inflammation and reduced weight gain. This study is particularly pertinent given the very low n-3:n-6 ratio in modern diets[191]; however, whether supplementation of n-3, reduction of n-6, or both is critical is unclear in this model. Additional questions remain as to the feasibility of achieving a high n-3 dose with concurrent reduction of n-6 in human pregnancy; maternal n-3 PUFA supplementation in healthy pregnant women had no effect on offspring adiposity or growth during early (up to 1 year of age) postnatal life[192,193].

Also looking to modulate obesity-associated inflammation, a recent study in rats used a cocktail of antioxidants (vitamins A, E and C and selenium) in conjunction with a HF or C diet. Antioxidant supplementation to HF-diets did not affect dam adiposity but did increase glucose tolerance. In the offspring at 2 months of age, adiposity in pups from HF-fed dams was reduced by antioxidant supplements and they exhibited significantly improved glucose tolerance, reduced serum leptin and normalised serum insulin[194]. Offspring oxidative stress, from the blastocyst stage through to neonates, was raised by maternal HF diet and significantly reduced by antioxidants. While this data is promising, human studies have failed to find evidence of antioxidant benefit to pregnancy outcomes. Two recent meta-analyses found evidence that vitamin C and E supplementation increased risk of gestational hypertension and did not reduce risk of preeclampsia in women at high risk of the condition[195,196]. One meta-analysis also reported increased risk of low birth weight with antioxidant supplementation[195]. Although these studies did not specifically assess obese pregnancies, these women were heavily represented in high preeclampsia risk subgroupings, which did not show any increased efficacy of the intervention. In the VIP (Vitamin C and vitamin E in pregnant women at risk for preeclampsia) randomized controlled trial including 2,410 women, supplementation of women at high-risk of preeclampsia (33% deemed so because of a BMI in first pregnancy >30kg/m^2) with vitamin E and A did not reduce rate of preeclampsia, but did significantly increase the incidence of small-for-gestational-age neonates[197]. These findings led the study authors to declare the use of antioxidants for the condition unjustified.

1.4.2 Pharmaceutical interventions

Research into pharmaceutical interventions during pregnancy to modulate alleviate developmental priming has been scant, primarily due to safety concerns including risk of teratogenicity. Roberts *et al.* supplemented the ONPRC NHP maternal obesity model with resveratrol, a putative SIRT1 activator. Although the drug reduced maternal weight, improved maternal glucose tolerance and decreased placental inflammation and fetal hepatic TAG

accumulation, fetal pancreatic mass was increased by 42%, leading the study authors to strongly caution against its use in humans. Glibenclamide, an antidiabetic drug in the sulfonylurea class, has been used in human pregnancy to treat GDM. However, glibenclamide is associated with increased risk of infant macrosomia and neonatal hypoglycaemia compared with metformin, with similar efficacy on maternal glucose management[198,199].

Statins have proven effective in a maternal hypercholesterolaemia model at reducing offspring body weight and cholesterol levels[200]. Data from animal and epidemiological studies has suggested that statin intake during pregnancy might associated with increased risk of fetal malformations, although a recent large, well-controlled epidemiological assessment found no increased risk. However, doubts remain about the safety of statins in pregnancy, and they remain contraindicated during pregnancy in the USA and UK and many other countries around the world[201].

However, the apparent safe use of metformin, a biguanide class antidiabetic, during pregnancy for the management of gestational diabetes has raised question into its long term impact on offspring health[202].

1.5 Metformin – impact on mother and child

Numerous studies have demonstrated the safety of metformin use from conception throughout pregnancy in obese women or those at risk of gestational diabetes, and the efficacy of the drug at reducing risk of adverse events associated with obesity and hyperglycaemia[203-207]. This is despite metformin freely, and potentially actively, crossing the placenta[208-210]. Its primary antidiabetic mechanism is the suppression of hepatic gluconeogenesis via a reduction in hepatic glucagon signalling[211], yet it may also reduce ROS production and improve oxidation of fatty acids in a mechanism dependent on AMPK activation[212]. Metformin has a very low risk of hypoglycaemic events[213], does not directly stimulate insulin secretion (unlike sulfonylureas), and has a greatly reduced risk of lactic acidosis compared to older biguanides (1 to 16.7 cases per 100,000 patient-years)[214].

1.5.1 Mechanisms of metformin action

Metformin exerts a range of antidiabetic effects, and has shown promise in treating symptoms of obesity (reviewed in[215]). The primary mechanism of metformin's blood glucose lowering effect is suppression of hepatic gluconeogenesis[216], resulting in decreased hepatic glucose production, with additional secondary systemic effects including (but not limited to) increased muscle glucose uptake[217] and suppression of hepatic DNL[218]. Metformin exerts a subtle yet specific inhibitory

influence on Complex I of the mitochondrial electron transport chain, resulting in decreased ATP synthesis and a corresponding increase in AMP:ATP ratio[219]. Although metformin was once thought to exert its anti-gluconeogenic effects solely through activation of AMPK as a result of increased AMP:ATP[220], it appears now that high concentrations of AMP downregulates cyclic AMP production at cell membranes[211]. Since glucagon, a major driver of hepatic gluconeogenesis, acts primarily by increasing membrane-based production of cyclic AMP, metformin interrupts glucagon signalling at the earliest cellular opportunity.

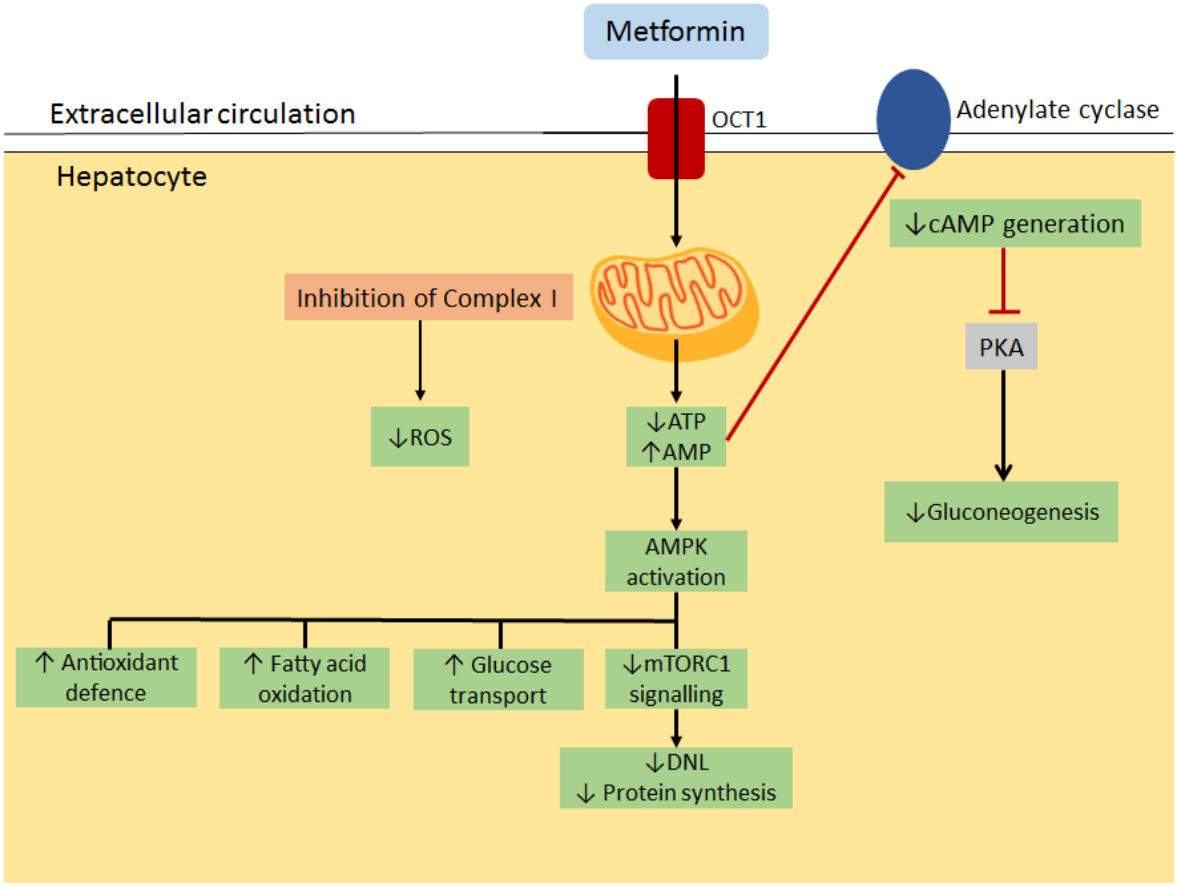

Figure 3 | **Primary effects of metformin in the hepatocyte.** The main glucose lowering effect of metformin probably stems from inhibition of adenylate cyclase, opposing the action of glucagon by preventing activation of protein kinase A (PKA). Increased levels of AMP also stimulate AMPK, which exerts numerous downstream effects beneficial for improving hepatic metabolism. AMPK, 5' AMP activated protein kinase; AMP, adenosine monophosphate; ATP, adenosine triphosphate; cAMP, cyclic AMP; DNL, *de novo* lipogenesis; mTORC1, mechanistic target of rapamycin complex 1; OCT1, organic cation transporter 1; PKA, protein kinase A; ROS, reactive oxygen species.

Indirect AMPK activation by metformin is however responsible for longer term transcriptional regulation of genes involved in glucose homeostasis, including: increased hepatic expression of SHP (small heterodimer partner), a potent inhibitor of G6Pase and PEPCK, pro-gluconeogenic genes[221]; disassembly of the pro-gluconeogenic CREB-CBP-CRTC2 complex via AMPK-induced PKC

ι/λ activation; the stimulation of TBC1D1-mediated GLUT4 translocation in striated muscle to the plasma membrane to facilitate glucose disposal[217]; and the AMPK-dependent activation of SIRT1), resulting in CRTC2 (a key co-activator of gluconeogenic gene expression) degradation via its deacetylation[222,223].

1.5.2 Metformin and lipid metabolism

In addition to direct glucose homeostasis modification, AMPK is involved in the regulation of lipid metabolism. Treatment with metformin is associated with improved blood lipid profiles and reduced hepatic lipid content in both animal[224,225] and human studies of TIIDM[226,227], in a mechanism thought to be dependent upon AMPK activation[220]. Activation of AMPK results in acetyl-CoA carboxylase (ACC) phosphorylation and inhibition, dramatically altering the metabolism of cellular fatty acids; ACC inhibition prevents formation of malonyl-CoA, a precursor to fatty acid synthesis and potent inhibitor of the rate-limiting fatty acid β-oxidation enzyme carnitine palmitoyltransferase I (CPT1). The subsequent lack of inhibition of CPT1 increases oxidation of fatty acids[228]. AMPK also inhibits the post-translational processing of the key lipogenic transcription factor SREBP1c[229], negating its activity, and down-regulates its transcription via negative regulation of the mTORC1 pathway in a TSC2-dependent manner[230]. As hepatic accumulation of the lipogenic intermediate DAG is independently associated with insulin resistance[19], modulation of hepatic lipid metabolism represents a secondary mechanism by which metformin could down-regulate hepatic gluconeogenesis.

1.5.3 Anti-inflammatory effects

There is some evidence that metformin exerts independent anti-inflammatory effects in addition to those mediated by its anti-hyperglycaemic anti-lipogenic properties. Activation of manganese superoxide dismutase (MnSOD), a critical antioxidant defence, was reported following metformin administration and blocked by an AMPK inhibitor[231]. Likewise, upregulation of the antioxidant thioredoxin by metformin seems AMPK dependent[232]. Metformin appears to reduce activation of the key pro-inflammatory transcription factor NF-kB in smooth muscle[233] and endothelial cells[234] in a mechanism dependent on AMPK. In contrast to other inhibitors of Complex I such as rotenone, metformin treatment results in decreased ROS production[235]. As increased ROS levels as a result of maternal nutrient excess tend to exacerbate dysfunction in fetal[74,154,236] and placental[237] tissue, there is scope for metformin having a beneficial effect on oxidative stress in the fetus and placenta. However, a degree of oxidative stress is essential for proper development, suggesting metformin should be used cautiously in mothers with low levels of ROS production, such as those without obesity[238].

1.5.4 Effects of metformin on offspring after maternal use

The effect of maternal metformin treatment during gestational diabetes on offspring long-term outcomes has been assessed in the Metformin in Gestational diabetes (MiG) trial, which has incorporated a follow-up of offspring at 2 years with further planned follow-up[239]. Briefly, 751 women with gestational diabetes at 20-33 weeks of gestation were randomized to either metformin (with supplemental insulin if needed) or isolated insulin therapy to manage blood glucose levels. Compared to insulin, there was no increase in any perinatal complications in the metformin treatment group, nor any differences in neonate physiology. However, 46% of the metformin group required supplemental insulin to achieve adequate blood glucose control. At the first two-year follow-up, offspring from the metformin group showed three significant differences to those born to insulin treated mothers. Upper arm circumference was significantly increased in the metformin treatment group, as was subscapular and bicep skinfold thickness, suggesting an increase in subcutaneous fat deposition in these areas. However, no differences were observed in total body fat content. This finding can be interpreted as a preferential storage of lipids in subcutaneous over visceral or ectopic depots, yet the study did not confirm this, nor is it apparent as to whether this adaptation would be metabolically beneficial in young children, as it is in adults[240].

Other trials have assessed the effect of maternal metformin treatment compared with insulin on short-term offspring outcomes. In a recent meta-analysis of trials comparing these drugs for the management of maternal TIIDM or GDM, 7 trials reported on LGA and SGA; metformin decreased risk of LGA (RR 0.80, 95% CI 0.64-0.99) but did not alter risk of SGA, and there was no increased risk of neonatal death or malformation[241]. Only one trial other than the MiG trial reported offspring follow-up. Ijas *et al.* reported that offspring of mothers with GDM randomized to metformin weighed more at 12 and 18 months of age, and were taller at 18 months of age, than offspring whose mothers received insulin, although there was no differences in body composition[242]. These data support the concept that metformin is safe to use during pregnancy, albeit based upon relatively limited offspring follow-up.

Two randomized placebo-controlled trials have investigated whether metformin can reduce fetal overgrowth occurring in obese pregnancy without GDM, with both showing no effect of metformin in reducing birth weight z score. In the EMPOWaR trial, women with obesity (mean BMI 37.7kg/m^2) received metformin (n =226) or placebo (n=223) from week 12-16 of gestation until delivery[243]. Although no effect on birth weight, or maternal GDM incidence (the main secondary outcome) was seen, maternal CRP and IL-6 plasma levels were reduced by metformin, and FBG and HOMA-IR were lower than in the placebo group at 28 weeks gestation (but not 36

weeks gestation). In the Metformin versus Placebo in Obese Pregnant Women without Diabetes Mellitus (MOP) trial, obese women (median BMI 38.7 kg/m^2) were given metformin (n=202) or placebo (n=198) at study entry between weeks 12 and 18 of gestation until delivery[244]. There was no difference in neonatal birth weight z score, although women taking metformin did gain less weight during gestation (median 4.6 kg vs 6.3 kg) and incidence of preeclampsia was significantly reduced (3% of women on metformin vs 11.3% of women on placebo). Thus, these data suggest that maternal metformin treatment commencing between gestational weeks 12-18 in obese pregnancy does not reduce birth weight; however, future protocols could focus on administration of metformin earlier in gestation (such as prescribed prophylactically to women looking to conceive), and the longer-term effects on offspring are currently unknown. The GRoW randomized placebo-controlled trial is currently assessing whether metformin can serve as an adjunct to lifestyle intervention for the prevention of fetal macrosomia[245].

To date, metformin use in animal models of maternal obesity and HF-feeding is limited to a handful of rodent studies. Tong and colleagues showed a range of impacts of maternal metformin supplementation during gestation and lactation on development of skeletal muscle in 60-day-old male offspring of obese, HF-fed dams[145]. Metformin treatment in offspring of obese dams reduced offspring gonadal fat pad mass, without significantly affect offspring bodyweight, a finding that may be related to increased skeletal muscle mass. Offspring of metformin treated obese dams also showed improved glucose tolerance and mitochondrial number and function compared to offspring of untreated dams. Whilst studies in humans suggest that skeletal muscle mitochondrial function impairment is not a primary cause of insulin resistance (reviewed elsewhere[246]) this evidence suggests metformin exposure during gestation may ameliorate the deterioration of mitochondrial function and activity seen in diabetic and insulin resistant states in later life[247,248]. Whether the persistent beneficial changes seen in these offspring are the result of a direct effect of metformin on fetal and neonatal development, a secondary effect of improvement of maternal obesity pathologies or a combination of both factors is unclear.

Maternal metformin treatment also appears able to abrogate maternal-obesity induced fetal inflammation. In a recent study in rats, obese, HF-fed dams were treated with metformin during gestation until E19. Maternal metformin did not affect measured maternal metabolic and inflammatory molecules, but did decrease the key inflammatory markers TNF and chemokine (C-C motif) ligand 2 (CCL2) in fetal plasma. Considering the apparent lack of impact on maternal characteristics, metformin may be directly reducing inflammatory markers in fetal circulation, a finding seen in non-pregnant human PCOS patients treated with the drug[249] and in isolated cell populations[250]. This is a potentially beneficial effect, given the major contributory role

inflammation seems to play in the developmental priming of several adult diseases, including NAFLD[251] and cardiovascular disease[252].

In an interesting investigation in a mouse model of the impact of metformin on normal weight mothers, offspring exposed to maternal metformin during pregnancy without the presence of maternal obesity were smaller at E18.5, indicating a growth restriction effect[253]. After birth, bodyweights of both males and females remained similar; however, when challenged with a HF-diet at 9 weeks of age, offspring of both sexes exposed to metformin gained dramatically more bodyweight, with significantly more mesenteric fat and tendencies towards increased fat deposition in other depots and reduced lean body mass. Male offspring alone exhibited impaired glucose tolerance and severely impaired fasting glucose after the HF-diet challenge when exposed to metformin *in utero*. It is interesting that the phenotype observed closely mirrors that seen in offspring exposed to maternal undernutrition. Metformin yields a physiological response similar to fasting[254], and may adversely affect delivery of nutrients to the developing fetus, or induce a thrifty phenotype response directly in fetal tissue as a result of perceived starvation. The same group also administered metformin to obese dams during pregnancy, and found that offspring had an altered response to postnatal HF diet[255]. After six weeks of HF diet, female offspring of metformin-treated obese dams had gained less weight and had less adipose tissue than female offspring of non-treated obese dams, and there were trends towards these effects also appearing in male offspring. Male and female offspring of metformin-treated obese dams also showed improved glucose tolerance. Together, these data highlight that metformin may be exerting effects well into the adult life of the offspring.

1.6 Conclusions

In summary, metformin represents an interesting proposition for the improvement of offspring outcomes in obese pregnancy. Data in (admittedly limited) human studies seem in good agreement: supplementation of obese women from 12-18 weeks gestation with metformin does not reduce obesity-related birth weight. However, the longer term outcomes of metformin in human offspring remain unknown. No follow-up has been conducted on offspring beyond 2 years of age in those born to mothers using metformin to control GDM, and no follow-up has been published at all on offspring born to obese mothers without GDM given metformin (although follow-ups are planned and forthcoming). Animal models suggest metformin has effects in adult offspring of treated mothers, both lean and obese. However, characterization of these offspring has been limited to investigations of whole-body adiposity, glucose homeostasis, skeletal muscle and adipose tissue; the effect of metformin on the adult liver, which experiences marked developmental priming by maternal obesity and is central to energy homeostasis, is unclear. In

addition, the effects of an earlier metformin intervention, such as one administered prior to or around conception, remain unclear. Intervention at earlier time periods has been suggested as necessary to improve treatment efficacy, and this approach may be explored in the future; animal models of metformin treatment in obese pregnancy have begun the treatment periconceptionally[145,253,255]. Furthermore, the effect of metformin in lean pregnancy remains underexplored. Salomaki *et al.* showed detrimental effects in offspring of lean dams receiving the drug at the same dose as obese dams, raising the question of whether this effect is conserved in other animal models and potentially in humans, and which aspect(s) of lean physiology result in negative offspring effects when metformin is given[253]. Further exploration of these deleterious effects is also warranted, as Salomaki *et al.* assessed only adult body weight, body composition, glucose tolerance and liver weight.

Chapter 2: Aims and Hypotheses

2.1 Aims

The aim of this thesis is to examine the effect of maternal metformin treatment during obese and lean pregnancy/gestation on dams, fetuses and adult offspring. Specifically, this thesis will assess the effect of metformin during pregnancy (both obese and lean) on the development in the offspring of the metabolic syndrome and associated comorbidities, notably NAFLD. A number of important characteristics have not yet been described in human studies or animal models of metformin in the context of maternal obesity. These unexplored parameters include the effect of metformin on offspring NAFLD risk and severity, which is known to be increased as a result of maternal obesity[66]. In addition, the effect of metformin on adult offspring hepatic lipid metabolism, a key feature in NAFLD, remains unknown, having only been assessed in fetal mice[256]. Importantly, it is vital that both male and female offspring are investigated, given the stark sexual dimorphism displayed as a result of developmental priming[257,258]. Moreover, only one study has assessed the effect of metformin in lean pregnancy on offspring[253], and this study only investigated glucose tolerance and body composition in the offspring.

Therefore, this thesis aims to address the following gaps in the extant literature:

- Generate a model of maternal metformin treatment incorporating lean and obese dams and assess both male and female adult offspring
- Investigate the effects of postnatal HF diet on offspring phenotype to determine whether observed changes are exacerbated in later life
- Assess phenotypic changes in older mice (30 weeks of age) than included in other published studies to better expose metabolic abnormalities only becoming apparent with age
- Explore the effect of maternal metformin treatment on offspring risk and severity of NAFLD

2.2 Hypotheses

2.2.1 Metformin improves maternal glucose homeostasis during obese pregnancy and mitigates obesity- and HF-diet-mediated effects on fetuses; metformin in lean pregnancy promotes a growth restriction fetal phenotype

The first hypothesis is that metformin treatment will improve maternal blood glucose homeostasis in obese dams during pregnancy, in line with data from human[198] and animal models [255]. Furthermore, although mouse models of maternal obesity can show fetal overgrowth or

undergrowth depending on strain and the gestational age of the fetus, as discussed in Chapter 1, metformin treatment is hypothesised to protect against altered fetal weight in obese pregnancy, as well as altered fetal liver size and expression of fetal genes related to energy metabolism, on the basis of its direct antihyperglycaemic effects on both mother and fetus. Given the data presented by Salomaki *et al.* showing deleterious effects of maternal metformin on adult offspring of lean dams[253], it is hypothesized that metformin in lean pregnancy suppresses fetal growth as a result of activation of catabolic pathways in the placenta and the fetus[259]. This hypothesis is supported by undernutrition models of pregnancy, which show exacerbated metabolic dysfunction in postnatal life in offspring exposed to inadequate maternal nutrition compared with offspring receiving adequate *in utero* nutrition[260-262], and the calorie-restriction-mimicking effect of metformin[254].

2.2.2 Maternal metformin reduces maternal-obesity-mediated priming of excess offspring adiposity, increased glucose intolerance, insulin resistance and NAFLD, in a sex-dependent manner

Data from mouse models demonstrate that offspring of obese dams typically have increased adiposity, impaired glucose homeostasis and insulin resistance. NAFLD, the hepatic component of the metabolic syndrome, is also made more severe in postnatal life by exposure to early-life overnutrition[66]. Importantly, these effects regularly manifest in sex-specific fashion when reported, yet many studies only report data on one sex[258]. Thus, the second hypothesis is that metformin, via direct effects on the fetus and/or via indirect effects on maternal obesity phenotype, primes protection against the effects of maternal obesity in the offspring.

Maternal obesity primes increased offspring adiposity, probably via alterations to hypothalamic appetite signalling and/or integration of signals influencing appetite from peripheral tissue[263], such as leptin signalling[150]. The exact mechanisms responsible for this priming are unknown, yet animal studies have demonstrated that maternal hyperglycaemia alone is able to increase offspring adiposity[264], and a wealth of human data supports a role for glucose in driving excessive fetal growth[265], which can track into increased adiposity in later life[266]. Moreover, the ability of maternal antioxidant supplementation to mitigate priming of offspring metabolic health by maternal obesity suggests a role for maternal redox homeostasis and possibly inflammation in mediating priming effects[194]. Metformin reduces hyperglycaemia and reduces ROS generation[267], so it is hypothesized that treating obese dams with the drug will counteract the priming effect of maternal obesity directly.

NHP fetuses exposed to maternal obesity *in utero* show marked hepatic lipid accumulation and evidence of oxidative stress, potentially priming development of NAFLD in later life. Acute metformin treatment increases fatty acid oxidation, supresses DNL, reduces hyperglycaemia (thus suppressing overnutrition *in utero*) and reduces inflammation[259,268], all beneficial properties in the context of early-life NAFLD priming. In addition, adult offspring of obese dams show impaired mitochondrial electron transport chain activity[269]. Metformin, via inhibition of ETC complex I, suppresses ROS formation[267], which is associated with hyperglycaemic-related damage to protein, DNA and lipid, potentially impairing mitochondrial function[270]. In addition, AMPK, which is activated by metformin, induces upregulated expression of antioxidant enzymes including MnSOD and catalase[271]. Metformin may therefore help to protect mitochondria from early priming events via both direct and indirect mechanisms. Thus, metformin is hypothesized to protect against the priming of more severe NAFLD by maternal obesity through direct effects on hepatic lipid accumulation and mitochondrial function in the fetus, and this might track in to adult life.

2.2.3 Maternal metformin increases the detrimental effect of a postnatal HF diet in offspring of lean dams, in a sex-dependent manner

Finally, it is hypothesized that maternal metformin treatment during lean pregnancy primes adverse metabolic responses in offspring, particularly when they are exposed to postnatal HF diet. In the absence of substantial opposing action from activators of mTORC1, mTORC1 signalling might be suppressed to pathological levels in lean dams by metformin, impairing fetal development directly or via reduced placental nutrient transfer. Given the resultant nutrient restricted state, it is hypothesised that metformin will promote offspring adiposity, upset glucose homeostasis and increase NAFLD severity and hepatic lipid accumulation, as a result of induction of a calorie-restriction-like state during gestation and mismatch between prenatal and postnatal environments[272]. Accordingly, these changes are likely to be exacerbated markedly by the effect of postnatal obesogenic diet.

Chapter 3: Methods

3.1 Animal model

All studies were conducted under UK Home Office License, and in accordance with the UK Animals (Scientific Procedures) Act of 1986. All C57BL/6 mice used in the studies were maintained under controlled temperature (20 ± 2°c) and humidity conditions, 12:12hr light-dark cycles (lights on at 7:00am), and *ad libitum* access to water, at the Biomedical Research Facility at Southampton General Hospital, Southampton. C57BL/6 are very commonly used for studies of metabolic disease as they become obese, glucose intolerant and insulin resistant in response to prolonged feeding of a human-palatable obesogenic diet, recapitulating the core aspects of human obesity and metabolic syndrome[273].

3.1.1 Generation of obese dams

Female C57BL/6 mice (who are proven breeders) were randomly assigned to receive either a custom high-fat diet (HF; 45% kcal fat, 20% kcal protein, 35% kcal carbohydrate; 824053 Diet, Special Diet Services, UK) or standard chow diet (C; 7.5% kcal fat, 17.5% kcal protein, 75% kcal carbohydrate; RM1, Special Diet Services), given *ad libitum* for 6 weeks prior to and then during mating, pregnancy and lactation, generating two maternal groups: C and HF. Identical HF diet provided *ad libitum* has previously been shown to induce weight gain in studies of maternal developmental programming [70,177] Energy content of the respective C and HF diets are listed in Table 1. Table 2Table 2 lists specific micro- and macronutrient compositions of the two diets. Dams were weighed every 2 weeks and subjected to a 2-hour intraperitoneal glucose tolerance test (IPGTT, described below) prior to mating to assess the impact of HF diet on glucose tolerance and fasting blood glucose.

	C	*HF*
%kcal from:		
Fat	7.4	45
Protein	17.5	20
Carbohydrate	75.1	35
kcal per gram	3.53	4.54

Table 1 | Energy content of diets used in this study. C, chow; HF, high fat.

SFAs	**C**	**HF**		**Macro minerals**	**C**	**HF**
C12:0 Lauric	0.02%	0.03%		Calcium	0.73%	0.63%
C14:0 Myristic	0.14%	0.31%		Phosphorus	0.52%	0.62%
C16:0 Palmitic	0.31%	4.28%		Sodium	0.25%	0.16%
C18:0 Stearic	0.04%	1.91%		Chloride	0.38%	0.28%
MUFAs				Potassium	0.67%	0.45%
C14:1 Myristoleic	0.02%	0.02%		Magnesium	0.23%	0.08%
C16:1 Palmitoleic	0.09%	0.03%		**Micro minerals**		
C18:1 Oleic	0.77%	6.43%		Iron	159.30 mg/kg	59.90 mg/kg
PUFAs				Copper	11.50 mg/kg	8.76 mg/kg
C18:2(ω6) Linoleic	0.69%	3.75%		Manganese	72.44 mg/kg	13.84 mg/kg
C18:3(ω3) Linolenic	0.06%	0.39%		Zinc	35.75 mg/kg	68.97 mg/kg
C20:4(ω6) Arachidonic	0.13%	0.01%		Cobalt	634.10 µg/kg	0
C22:5(ω3) Clupanodonic	0%	0%		Iodine	1202.69 µg/kg	254.84 µg/kg
Amino acids				Selenium	298.99 µg/kg	197.40 µg/kg
Arginine	0.91%	0.73%		Fluorine	10.49 mg/kg	1.24 mg/kg
Lysine	0.66%	1.46%		**Vitamins**		
Methionine	0.22%	0.57%		β-Carotene	0.16 mg/kg	Not given
Cystine	0.24%	0.45%		Retinol	2.56 mg/kg	Not given
Tryptophan	0.18%	0.20%		Vitamin A	8554.27 iu/kg	4936.44 iu/kg
Histidine	0.35%	0.53%		Cholecalciferol	15.54 µg/kg	Not given
Threonine	0.49%	0.80%		Vitamin D	621.7 iu/kg	1416 iu/kg
Isoleucine	0.54%	1.16%		α-Tocopherol	76.45 mg/kg	Not given
Leucine	0.98%	1.76%		Vitamin E	84.10 iu/kg	100.72 iu/kg
Phenylalanine	0.66%	0.96%		Vitamin B1	8.58 mg/kg	6.10 mg/kg
Valine	0.69%	1.39%		Vitamin B2	4.33 mg/kg	6.37 mg/kg
Tyrosine	0.49%	0.96%		Vitamin B6	4.81 mg/kg	7.04 mg/kg
Taurine	0%	0%		Vitamin B12	7.49 µg/kg	30.85 µg/kg
Glycine	1.11%	0.91%		Vitamin C	2.59 mg/kg	0
Aspartic Acid	0.67%	1.30%		Vitamin K	10.17 mg/kg	0.95 mg/kg
Glutamic Acid	3.17%	3.77%		Folic acid	0.79 mg/kg	2.38 mg/kg
Proline	1.20%	1.58%		Nicotinic acid	61.32 mg/kg	36.65 mg/kg
Serine	0.56%	0.87%		Pantothenic acid	20.17 mg/kg	18.48 mg/kg
Hydroxyproline	0%	0%		Choline	1080.14 mg/kg	1392 mg/kg
Hydroxylysine	0%	0%		Inositol	2369.59 mg/kg	0
Alanine	0.16%	0.74%		Biotin	277.13 µg/kg	246.82 µg/kg

Table 2 | **Specific micro- and macronutrient composition of chow and high-fat diets.** Data obtained from Special Diet Services (SDS). C, chow; HF, high-fat; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

3.1.2 Mating and maternal metformin supplementation

The full experimental protocol is outlined in Figure 4. C and HF-fed dams were mated overnight with C-fed C57BL/6 males, with pregnancy confirmed visually by vaginal plug presence. Plug observation was designated as day 0.5 of gestation (E0.5). Males were then removed. 250mg/kg/day of metformin (Sigma-Aldrich, UK), corresponding to a final concentration of 1.75mg/ml, was administered orally in drinking water *ad libitum* to a subset of HF (HFm) or C (Cm) fed dams from plug confirmation until culling at gestational day 16 (E16.5) in study 1, or until weaning in study 2, generating four maternal groups: C, Cm, HF and HFm. This dose has previously shown physiological effects relevant to human doses in other rodent studies and is well-tolerated with no evidence of toxicity[274,275]. Moreover, concentrations in this dosage range have previously been shown to yield plasma metformin concentrations similar to those seen in humans[255]. To ensure adequate maternal metformin dosage in our model, we assessed plasma and amniotic fluid metformin concentrations by mass spectrometry (Chapter 4).

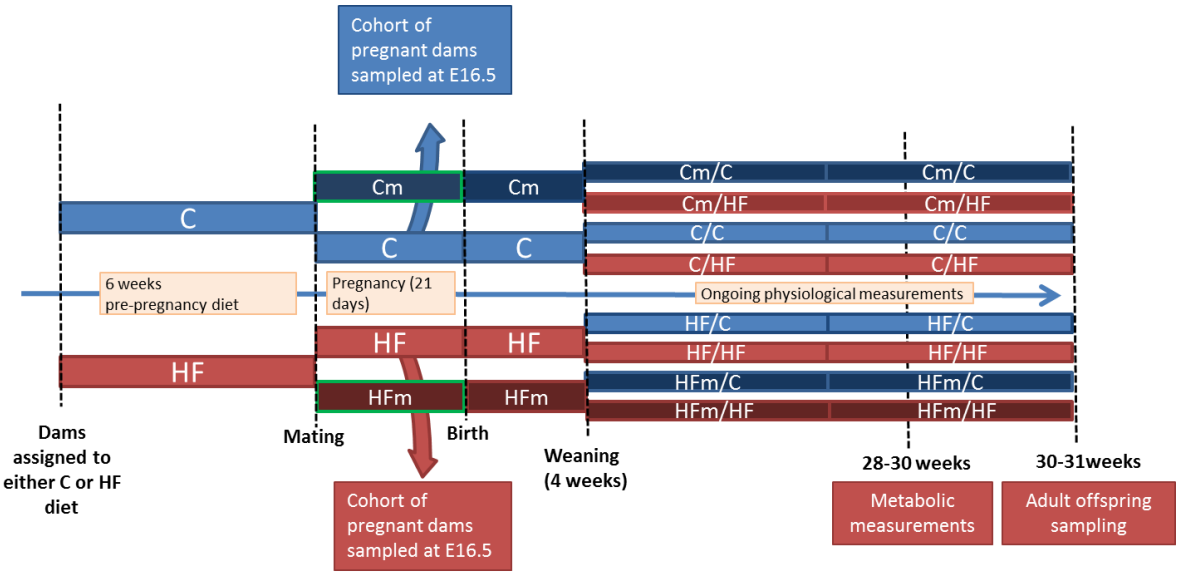

Figure 4 | Schematic of experimental protocol. C, chow diet; E16.5, day 16.5 of gestation; HF, high-fat diet; m, metformin treatment.

3.1.3 Quantification of metformin concentration

Concentration of metformin in dam plasma and amniotic fluid taken during tissue sampling at E16.5 was assessed by collaborators at AstraZeneca, UK, using laser diode thermal desorption/atmospheric pressure chemical ionization tandem mass spectrometry (LDTD-APCI-MSMS) as described in[276]. Briefly, dried samples were loaded into the LDTD system (Phytronix Technologies, Quebec, Canada) on a specially designed LazWell™ 96-well plate (Phytronix

Technologies). The LDTD source used the following settings: corona discharge needle voltage 3000 V, vaporizer temperature ambient, ion sweep gas pressure 0.3, auxiliary gas off, sheath gas off. The carrier gas was nitrogen at a flow rate of 3 l/min. Laser power was ramped from 0% to 35% over 3 s and held at 35% power for 3 s before shutting off.

The LDTD source was mounted on a Quantum Ultra mass spectrometer (Thermo Fisher Scientific, San Jose, California, USA). The mass spectrometer was operated in positive ion selected reaction monitoring mode. Metformin was monitored at a parent mass of 130.097 and a daughter mass of 71.14 with a tube lens voltage of 54.56 V and a collision energy of 22 V.

All data was processed using QuickQuan™ (Gubbs Inc., Alpharetta, Georgia, USA) software. Linear least-squares regression with a 1/*x* weighting of the peak area ratios (analyte/IS) versus the nominal concentration of the calibration standards was used to construct the calibration curves. Eight calibration standards between 1 and 2000 ng/ml (*n* = 6 at each level) were prepared.

3.1.4 Study 1: Impact of maternal obesity and metformin treatment on maternal physiology and fetal

To assess the impact of maternal HF feeding and obesity and metformin treatment on both maternal metabolic characteristics and fetal development, two subsets of pregnant dams, one set fasted overnight and one set unfasted, were culled at gestational day 16.5 (E16.5).

3.1.4.1 Fasted Dams

Fasted dams (C, n=4; Cm, n=4; HF, n=4; and HFm = 4) were subjected to a 2-hour IPGTT as described in Chapter 3, below. Details related to maternal and fetal samples from this group of dams are described in Methods in Chapter 4.

3.1.4.2 Unfasted Dams

Unfasted pregnant dams from each treatment and dietary group (C, n=5; Cm, n=3; HF, n=9; and HFm, n=7) were killed on E16.5 and maternal and fetal tissue and measurements obtained as describe in detail in Chapter 4

3.1.5 Study 2: Impact of maternal obesity and metformin treatment on adult offspring cardiometabolic function

A cohort of pregnant dams from each dietary treatment group (C, n=9; Cm, n=5; HF, n=8; and HFm, n=9) were allowed to give birth naturally and nursed pups until 4 weeks of age. Litter size was not arbitrarily adjusted by culling pups, as this intervention can exert deleterious and

unpredictable effects on offspring postnatal growth and physiology[277,278]. Instead, litter size was controlled for by including litter size as a covariate and treating individual litters as the experimental unit (via a mixed model approach) during statistical analysis (discussed later)[279]. 4-week-old offspring were weaned onto either C or HF diet, generating offspring in 8 diet/treatment groups (designated by maternal diet/postnatal diet and maternal metformin treatment exposure): C/C, Cm/C, C/HF, Cm/HF, HF/C, HFm/C, HF/HF and HFm/HF. All offspring were weaned into single-sex cages with at least one cage-mate. Each offspring group contained offspring from multiple litters to minimise litter-induced variation, and at least 5 litters-of-origin were planned for.

All offspring were subjected to a number of *in vivo* measurements between 26 and 30 weeks of age, as well as continuous monitoring of bodyweight; details of these procedures are described in full below. Female offspring oestrous stage was determined prior to cardiometabolic experiments and death using vaginal cytology as described in[280], and experiments were timed to coincide with proestrous to minimize potential variance induced by circulating sex hormone levels. At 27 weeks of age, offspring blood pressure was measured. At 28 weeks of age, offspring were placed in a metabolic cage to measure activity and energy expenditure, although this data became corrupted and unusable. IPGTTs were performed at 29 weeks of age. At 30-31 weeks, offspring were fasted overnight, then anaesthetised with isoflurane at 10-11am the following day. Cardiac puncture was performed to obtain blood (which was placed in heparinised tubes before separation of plasma), and death was confirmed by cervical dislocation. Body composition was then assessed (details provided below), and tissue taken and snap frozen. Weights of tissues were also recorded. Extremely haemolysed plasma samples were discarded.

3.2 Metabolic measurement procedures

3.2.1 Assessment of body composition

After cardiac puncture and confirmation of death by cervical dislocation at sampling, offspring were scanned using a Skyscan 1176 *in vivo* micro-tomography CT scanner at 35µm resolution (Bruker Corporation, Belgium). The current was 500µA and the voltage was 50kV, with 0.5mm aluminium filter. An image was taken every 0.1° of rotation for 180° rotation. The images were reconstructed into a 3D volume using NRecon software (Skyscan, Belgium). The reconstructed volume was analysed using VGStudio Max version 1.2.1. The x-ray absorbance of the volume produced three peaks corresponding to fat, soft tissue, and bone. The total volume of fat, soft tissue, and bone was determined by setting the thresholding limits to contain the relevant absorbance peak (grey scale 11-20 for fat, 21-40 for soft tissue, and 41-255 for bone). The volume

was then determined by the software. Total volume of the scanned region was the sum of all three volumes.

3.2.2 Collection of tissue samples

After confirmation of death, the following tissues were removed, weighed and snap frozen in liquid nitrogen: heart (whole heart weighed, left ventricle frozen only), liver (whole liver weighed, left lobe frozen only), pancreas, brain, skeletal muscle (gastrocnemius) and body fat (white adipose tissue (WAT: gonadal, retroperitoneal, perirenal and inguinal fat pads) and interscapular brown adipose (iBAT)). Left liver lobes were collected as maternal nutrition can differ depending on the lobe assessed, and the left liver lobe has previously been shown in rats to be more severely affected by maternal nutritional insults[281]. Left liver lobe tissue was fixed in 10% formalin for 24 hours then transferred to 70% ethanol and embedded in paraffin wax for histology and immunohistochemistry.

3.2.3 Intraperitoneal glucose tolerance tests

Mice to be subjected to IPGTT were fasted overnight (between 5pm and 9am). Basal fasted blood glucose concentration was obtained from tail blood using a glucometer (Accu-chek Aviva, Roche Diagnostics, Switzerland) following locally anaesthetised tail-tipping. Animals were then injected intraperitoneally with 20% glucose solution (2g/kg body weight) and blood glucose concentrations were further obtained at 15, 30, 60 and 120 minutes. Area under the curve (AUC) of the time taken to dispose the glucose bolus was calculated using GraphPad Prism 6 (GraphPad Software, Inc, California, USA) to assess glucose tolerance.

3.2.4 Measurement of blood pressure

Blood pressure was measured using the CODA tail cuff plethysmography system (Kent Scientific, Connecticut, CT) according to manufacturer's instructions. Mice were acclimatized in their own cages 30°C ambient temperature in a warm room for at least 30 minutes before measurements were made. Mice were restrained in restraint tubes (Harvard Apparatus, MA, USA) specific for their weights and allowed to acclimatize to restraint for 1 minute. Although the requirement for weight-specific tubes precluded blinding of the researcher to the obesity state of the animal, cage labels were removed so that the exact experimental identity of the animal was unknown during data collection. Heart rate and visual cues were used to assess animal stress, and animals that struggled against the restraint or had rapidly rising heart rates were immediately freed, returned to their cages, and repeated measures were attempted between 1 h and 24 h later. Maximum

occlusion pressure was set at 300mmHg and sensor cuff pressure at 90mmHg. Measurements were repeated until five individual readings were obtained. The highest and lowest readings were discarded and mean systolic and diastolic blood pressure obtained for each animal.

3.3 Analysis of collected tissue

3.3.1 Gene expression analysis

Total RNA was extracted from liver tissue samples by homogenising tissue using a handheld tissue homogeniser (Stuart SHM1, Stuart, UK) and Tri reagent (Sigma-Aldrich). Separation of RNA was performed with 1-bromo-3-chloropropane (Sigma-Aldrich), before precipitating with isopropanol and washing with 75% ethanol and resuspending in ultrapure RNase free water (Sigma-Aldrich). Concentration of extracted RNA samples was assessed by NanoDrop spectrophotometer (NanoDrop 1000, Thermo Scientific, USA). Purity of extracted samples was also assured by recording absorbance ratios at 260nm and 280nm (A_{260}/A_{280}) and at 260nm and 230nm (A_{260}/A_{230}), quantifying contamination by protein and salts/organic compounds respectively. Samples significantly contaminated were re-extracted. Aliquoted RNA was frozen and stored at -80°C.

cDNA synthesis was performed according to manufacturer supplied protocols using 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase per reaction mix (M-MLV RT; Promega, UK) with random primers (500uM, Promega) and 1000ng of RNA template. Reaction mixes were prepared in individual 500µl sealed tubes. Reaction mixes were incubated for 60 minutes at 37°C, and resultant cDNA was diluted to 5ng/µl, aliquoted and stored at -20°C.

Real-time qPCR was then performed using pre-optimised gene of interest Taqman probes, primers and reagents (Eurogentec, UK and PrimerDesign) and an ABI7500™ real-time PCR system (Applied Biosystems, USA). Mastermix containing GoTaq hot start polymerase was obtained from PrimerDesign. Each sample was plated in duplicate wells, and each well contained 20 ng of template cDNA. Amplification protocol for all genes (optimized for Taq polymerase and Taqman primers and probes from these manufacturers and on this PCR machine) was: 2 min at 95°C, followed by 40 cycles of 10 s denaturation at 95°C then 60 s data collection (via FAM fluor channel) at 60°C. A previously validated housekeeping gene in this model[282], *YWHAZ*, was used to normalise gene of interest data using the ΔΔCt method [283]. There were no statistically significant differences in the expression of *YWHAZ* between experimental groups.

3.3.2 Measurement of mitochondrial copy number

Total genomic DNA (gDNA) was extracted from liver tissue to assess mitochondrial copy number. Total gDNA was isolated from 20mg of homogenized liver using a commercial kit, Quick-gDNA MiniPrep, according to the manufacturer's instructions (Zymo Research, USA). Concentration and purity of extracted samples was assessed by NanoDrop, as described above. PCR was performed on total gDNA as described above, using primers for *YWHAZ* and a gene specific to the mitochondrial genome, NADH dehydrogenase 4 (*Nd4*). Mitochondrial copy number was then calculated as the ratio of respective Ct values between *Nd4* and *Ywhaz* for each sample. This method of calculating estimated mitochondrial copy number by the ratio between nuclear and mtDNA has been reported many times previously[284].

3.3.3 Processing of liver tissue for histological analysis

Left liver lobe sections were fixed immediately after death in excess 10% neutral buffered formalin for 24 h before placed into 70% ethanol. Samples were then embedded in paraffin, 4μm sections were cut on a Leica RM2125 microtome, and sections were mounted on clear slides (three nonadjacent sections per slide).

3.3.3.1 Liver haematoxylin and eosin staining

One slide per animal was stained with haematoxylin and eosin (H&E) for assessment of hepatic morphology and NAFLD activity scoring according to a standard protocol. Briefly, sections were dewaxed in clearene and hydrated through graded alcohols (100% ethanol, 70% ethanol) and double distilled H_2O. Sections were then stained in Mayer's haemotoxylin for 5 minutes, washed in running tap water for 5 minutes, and then stained in eosin for 5 minutes. Sections were then dehydrated (through 70% ethanol, clearene and xylene) and mounted with Pertex mounting media (Histolab, Sweden).

3.3.3.1.1 Quantification of hepatic steatosis on H&E stained slides

Hepatic steatosis was assessed in H&E stained slides by estimating the area of the observed section occupied by steatotic vesicles. Briefly, images of sections were captured at 200 x total magnification using a Nikon Eclipse E600 photomicroscope and attached 4MP digital camera (Nikon, Japan). 10 images per animal were taken. Images were taken consecutively at the same camera zoom, with no overlap between fields, from left to right across the section; upon reaching the right-most edge of the section, the field was moved down and another row of images were collected from right to left, again ensuring no overlap with previously captured fields. This process was repeated until 10 images were obtained. Images were not taken from adjacent liver sections.

File names of the images were recorded against the experimental identity of the animal. These file names were then randomized by an external individual who kept the identity of the animals hidden until the analysis of collected data.

Images were batch imported into ImageJ version 1.50 (National Institutes of Health). A 10 x 10 grid of points was overlaid on each image. A point was counted as a "hit" if it lay within a steatotic vesicle. The number of hits divided by the total number of points (100) multiplied by 100 therefore represents an estimate of the total section area, as a percentage, occupied by steatotic vesicles. The mean percentage area occupied by steatosis was calculated for each animal from all 10 section images. Identities of the images were then unblinded to allow statistical analysis.

3.3.3.1.2 Semi-quantitative analysis of NAFLD severity

Using a Nikon Eclipse E600 photomicroscope at 200 x total magnification, sections (blinded and randomized before analysis) from each animal were scored for NAFLD severity using the NAFLD Activity Score, developed by Kleiner *et al.*[285]. This index uses the scoring criteria detailed in Table 2 below:

Factor	Score	Extent
Steatosis	0	<5% by area
	1	5 to 33%
	2	33% to 66%
	3	>66%
Lobular inflammation	0	No inflammatory foci
	1	<2 foci per 200x field
	2	2-4 foci per 200x field
	3	>4 foci per 200x field
Hepatocyte ballooning	0	None
	1	Few ballooned cells (includes presence of cells diagnostically borderline)
	2	Many cells and prominent ballooning

Table 3 | **Components of the NAFLD activity score (NAS) scoring system.**

The NAS thus generates a score between 0 and 8. Scores between 0 and 2 are considered, in humans, not diagnostic of NASH or NAFLD; scores 3 and 4 are considered NAFLD or borderline NASH, and scores between 5 and 8 are considered NASH[285]. Although this scoring system was not designed for use in mouse models of NAFLD, and was developed to monitor disease changes during clinical trials rather than for NAFLD diagnosis, the index has been used with success in preclinical research in animals, with the caveat that data derived are relative to the model used, rather than absolute and directly comparable with human NASH[70,286,287]. Mean total NAS and the mean value of each individual component was calculated for each experimental group.

3.3.3.2 Liver sirius red staining

One slide per animal was stained with sirius red stain for the assessment of hepatic collagen deposition, and thus hepatic fibrosis, according to a standard protocol. Briefly, sections were dewaxed in clearene and hydrated through graded alcohols (100% ethanol, 70% ethanol) and ddH_2O. Sections were then stained in sirius red solution for one hour, washed in acidified water and then dehydrated in 100% ethanol. Sections were then cleared in xylene and mounted with Pertex.

3.3.3.2.1 Quantification of hepatic fibrosis

Briefly, images of sections were captured at 200x total magnification using a Nikon Eclipse E600 photomicroscope and attached 4MP digital camera (Nikon, Japan). 10 images per animal were taken at the same camera zoom, with no overlap between fields, from left to right across the section; upon reaching the right-most edge of the section, the field was moved down and another row of images were collected from right to left, again ensuring no overlap with previously captured fields. This process was repeated until 10 images were obtained. Images were not taken from adjacent liver sections. File names of the images were recorded against the experimental identity of the animal. These file names were then randomized by an external individual who kept the identity of the animals blinded until the analysis of collected data.

For each of the 10 images taken per animal, hepatic fibrosis severity was scored according to the NAS fibrosis staging score[285]. This index grades fibrosis severity by the criteria listed in Table 3.

Fibrosis stage	Extent of fibrosis
0	No fibrosis
1	Perisinusoidal or periportal
1A	Mild zone 3 perisinusoidal
1B	Moderate zone 3 perisinusoidal
1C	Portal/periportal
2	Portal/periportal AND perisinusoidal
3	Bridging fibrosis
4	Cirrhosis

Table 4 | **Estimation of fibrosis stage criteria.**

Mean fibrosis staging score was calculated for each animal from the 10 images scored, and the mean group fibrosis stage was calculated. Grade 1A was scored 1.25, 1B was scored 1.5 and 1C was scored 1.75. Scoring all grade 1 subgrades (1A, 1B and 1C) as 1 had no effect on final results.

3.3.4 Characterization of hepatic lipid composition

Liver samples were analysed by gas chromatography according to a protocol similar to that previously reported[288]. 100mg of frozen left liver lobe was homogenized using a handheld tissue homogeniser (Stuart) in 800μl of ice cold 0.9% NaCl. Homogenate was then vortexed thoroughly with 2:1 chloroform/methanol containing butylated hydroxytoluene (50mg/l). Internal standards were added (TAG internal standard: 1.5mg in postnatal HF samples, 750μg in postnatal C samples;

cholesterol ester (CE) internal standard: 500μg in postnatal HF samples, 100μg in postnatal C samples; phosphatidylcholine (PC) internal standard: 200μg in all samples; NEFA internal standard: 50μg in internal standards). Lipid fractions were then separated by solid-phase extraction on aminopropylsilica cartridges (Kinesis, Cambridgeshire, UK). CEs and TAGs were eluted with chloroform. PC was eluted with 3:2 chloroform/methanol. NEFAs were eluted using 50:1:1 chloroform/methanol/glacial acetic acid. After binding to a new cartridge, CEs were separated from TAGs by eluting with hexane; TAGs were eluted with 100:5:5 hexane/chloroform/ethyl acetate. All lipid fractions were then dried under nitrogen, dissolved in toluene, and then heated at 50°C for 2 h with methanol containing 2% by volume H_2SO_4 to form fatty acid methyl esters (FAMEs). Neutralization was then achieved using $KHCO_3$. FAMEs were redissolved in hexane, dried under nitrogen, redissolved in a small amount of hexane and separated by gas chromatography. Samples were run on a Hewlett-Packard 6890 gas chromatograph fitted with a BPX-70 (30m x 0.22mm x 0.25μm) with helium as the carrier gas; inlet temperature was set at 300°C and oven temperature was 115°C for the first 2 minutes after injection. Oven temperature increased at a rate of 10°C per minute until reaching 200°C, followed by a hold at 200°C for 16 minutes, and an increase to 240°C at a rate of 60°C per minute, followed by a hold at 240°C for 2 minutes. Total run time was 29 minutes. FAMEs were detected and quantified using a flame ionization detector at a temperature of 300°C. HPChemStation (Hewlett-Packard, USA) was used to control the instrument and collect and analyse data. Retention times of previously run authentic standards were used to identify FAME species in samples.

3.3.5 Plasma analytes

Aliquots of plasma were sent to the Core Biochemical Assay Laboratory, Cambridge, UK, for analysis. Plasma was analysed for the concentration of the cytokines and metabolites listed in Table 5.

Metabolite	Protein
TAG	IFNγ
NEFA	IL-10
Total cholesterol	IL-12p70
β-hydroxybutyrate	IL-1β
	IL-2
	IL-4
	IL-5
	IL-6
	CXCL1
	TNF
	CRP

Table 5 | **Plasma metabolites and cytokines assessed.** NEFA, nonesterified fatty acid; TAG, TAG.

3.3.5.1 Measurement of plasma cytokines

Plasma cytokines, with the exception of CRP, were measured using a commercially available 10-plex electrochemical luminescence immunoassay (V-Plex 10-plex mouse pro-inflammatory cytokine kit; MesoScale Discovery, Gaithersburg, Maryland, USA) on a MesoScale Discovery Sector 6000 analyser. All reagents and calibrators were supplied with the kit and the assay protocol was set up according to manufacturers instructions.

Plasma CRP was measured using an in-house Dissociation-Enhanced Lanthanide Fluorescent Immunoassay (DELFIA) protocol specific to CBAL, but using reagents from an R&D systems DuoSet assay kit (Minneapolis, USA). Data was recorded on a Perkin Elmer Victor3 time-resolved fluorescence plate reader (Massachusetts, USA).

3.3.5.2 Measurement of plasma metabolites

Plasma β-hydroxybutyrate was measured using a Stanbio β-hydroxybutyrate Liquicolour Kit (Stanbio, Texas, USA) modified to run on a microtitre plate.

Plasma insulin was measured using a MesoScale Discovery kit (Gaithersburg, Maryland, USA). Plates were read on an MSD Sector 6000 plate reader.

Plasma TAG concentration was measured using a commercially available colourimetric enzymatic assay (Siemens Healthcare, Germany), with absorbance measured on a Siemens Dimension RxL analyser.

Plasma NEFAs were measured using a commercially available Roche Free Fatty Acid kit run on a microtitre plate.

3.4 Statistical analysis

3.4.1 Analysis of dam parameters

Because each dam used for breeding came from separate litters, mixed model analysis (discussed below) was not required. Before beginning metformin treatment, t-tests were used to assess the impact of maternal HF diet on dam bodyweight. For testing between the four maternal groups (C, HF, Cm and HFm), 2-way ANOVA was used, with maternal diet and maternal metformin treatment as main effects and the interaction between the main effects (diet*metformin) reported if significant.

3.4.2 Analysis of offspring parameters (fetal and adult)

The two-way analysis of variance (ANOVA) statistical test has a number of inherent weaknesses that mean it is inappropriate for use in the analysis of offspring data in this thesis.

Foremost is the assumption in the 2-way ANOVA of independence between offspring. Siblings from the same litter exposed to the same treatment (in this case maternal diet and/or maternal metformin) violate this independence assumption; those born to the same dam and weaned together are more likely to be similar than offspring born to different dams and weaned separately, owing to shared treatment exposure. As a result, when using two-way ANOVA as a method of statistical analysis, the litter (or the dam) should be regarded as the experimental unit, rather than the offspring. Use of multiple offspring from the same litter is likely to provide an unrealistically low degree of within-group variation. This problem can be avoided if using only one animal per litter, but this is not an efficient use of animals in a multiparous species. Averaging data from litters and proceeding with two-way ANOVA is another approach, but this risks amplifying the size of the litter-specific variation. It follows that litter-specific variation can introduce experimental ‘noise’ that masks true treatment effects.

To avoid these issues, the use of mixed models that incorporate estimation of random effects (by incorporating litter-of-origin in the model and allowing subjects to have different intercept values)

alongside fixed effects (such as the effect of maternal metformin treatment) have been advocated[279,289,290]. Moreover, they allow the structuring of data such that independence between subjects is not assumed, and their robustness is greater when dealing with groups containing different numbers of subjects. The ability to incorporate multiple offspring from the same litter within the analysis improves the power of the model as it incorporates all available experimental data.

Prior to statistical modelling, all dependent variables were assessed for normality. This was achieved by visually inspecting Q-Q plots and histograms, and assessing skewness (absolute skew value >2 approximately illustrates a substantial departure from normality) and kurtosis (reported adjusted kurtosis of ±2 approximately illustrates a substantial departure from normality) of data as reported by SPSS 22 (IBM Analytics, USA)[291]. Formal statistical tests of normality (such as the Kolmogorov-Smirnov test) have low power to detect normality in small samples sizes often encountered in biological science[292]. For samples found to be non-normal, data was log transformed and assessed again[293]. Handling of individual dependent variables is discussed in relevant chapters.

Hierarchical data was modelled in SPSS 22 using the MIXED procedure. Fixed effects (the so-called main effects of maternal diet, maternal treatment and postnatal diet (for adult offspring), and all possible interactions between them) were dummy coded 0 or 1. Litter-of-origin was included as a random effect to account for between-litter variation and within-litter similarities. Maximal models were constructed containing all main effects and their interactions. For adult offspring, this model comprised seven fixed effects: 1) maternal diet, 2) maternal metformin treatment, 3) postnatal diet, 4) maternal diet*maternal metformin treatment, 5) maternal diet*postnatal diet, 6) postnatal diet*maternal metformin treatment, 7) maternal diet*postnatal diet*maternal metformin treatment. For fetal offspring, this maximal model contained only 3 fixed effects: 1) maternal diet, 2) maternal metformin treatment and 3) maternal diet*maternal metformin treatment. Models were then simplified stepwise by removing the highest level interaction terms sequentially if these terms were not significant (that is, the three-way interaction term was removed first, followed by the least significant two-way interaction term, and so on). In addition, litter size as a continuous variable was also included as an additional fixed effect to account for potential litter effects owing to the non-standardization of litter sizes.

Effect size estimates, standard errors and *P* values from each selected model were obtained from SPSS mixed effect model outputs for each dependent variable analysed. The estimate of the fixed effect can be considered analogous to β coefficients in regression modelling.

Because offspring studied often came from similar litters, reporting raw observed data could result in inaccurate conclusions about the size of standard error; for example, offspring in the same experimental group from the same dam are more likely to exhibit less variation than offspring in the same experimental group from different dams. As a result, adjusted group means and standard errors are reported to account for these errors. This was achieved by constructing a maximal model containing all fixed effects and interactions, and generating predicted values for the eight experimental offspring groups (for adult offspring) or the four experimental offspring groups (for fetal offspring) for a given dependent variable. Coding of the dummy variables for this maximal model was altered to enable comparison between metformin-treated experimental groups and their respective non-metformin-treated experimental groups, providing four planned comparison tests of the group means to assess the effect of metformin between pairs of groups. These tests were not adjusted, and to avoid type I error caution should be, and has been, exercised when discussing these tests.

Predicted means and standard errors (thus controlling for the effect of multiple offspring potentially coming from the same litter, in contrast to observed means and standard errors) for all dependent variables were graphed in GraphPad Prism.

Chapter 4: Acute effects of maternal obesity and metformin treatment

4.1 Introduction

The link between maternal obesity and overweight and risk of acute and chronic risk of disease to the offspring is well-established. Maternal obesity not only increases risk of congenital birth defects, still birth and early neonatal death, but also increases offspring propensity to metabolic disease in later life in both animal models[152,294,295] and human studies[296-299]. The impact of maternal obesity on offspring solely as a result of *in utero* exposure prompts investigation of how mothers and fetuses respond in the short-term when exposed to obesity, and how maternal interventions affect these responses. Experiments in human fetuses are severely restricted by ethical concerns to minimally invasive observational studies and characterizations of the neonate immediately after birth, and as a result the majority of data on the fetal impacts of maternal obesity are derived from animal studies. Despite a number of rodent studies reporting alterations to pup birth weight as a result of maternal obesity and high-fat (HF) diet feeding[160,300], indicating disrupted development, relatively few studies have investigated the programming of developing fetal tissues with respect to adult predisposition to disease, and fewer still have characterized how interventions influence maternal and fetal phenotypes.

Maternal obesity and HF diet in mice primes an increased risk of NAFLD and NASH in offspring, independent of the postnatal diet[70,286]. In humans, a single prospective study has found increased risk of NAFLD in adult offspring of mothers with gestational diabetes, independent of offspring adiposity, suggesting persistent, specific derangements to hepatic metabolism[67]. Human neonates born to obese mothers with gestational diabetes have increased levels of intrahepatic fat[68], suggesting early upset of hepatic metabolic pathways, and these findings have been also observed in fetal nonhuman primates[74].

Impairment of hepatic mitochondrial function has been demonstrated in a number of studies in offspring of obese mothers. Mitochondrial electron transport chain dysfunction in mice exposed to maternal obesity/HF-diet was noted by Bruce and colleagues, and was associated with increased risk of NAFLD and hepatic lipogenesis[70]; Burgueno *et al.* also showed a decrease in mitochondrial copy number in adult rats born to obese mothers[72], a finding seen in human NAFLD patients[301]. Mitochondria are maternally inherited, providing a potential direct link between maternal and fetal phenotypes, and are especially sensitive to damage induced by excessive ROS production, which typically occurs in states of overnutrition[152]. The regulation of both mitochondrial number and capacity for oxidative phosphorylation is strictly controlled and tied to cellular metabolic requirements, suggesting priming of these mitochondrial regulatory networks by maternal obesity could persistently affect mitochondrial function[75]. The epigenetic modulation of certain mitochondrial genes (NADH dehydrogenase 6 and cytochrome C oxidase I) has been

associated with the histological severity of NASH, and the switch between simple steatosis and NASH, in humans[302].

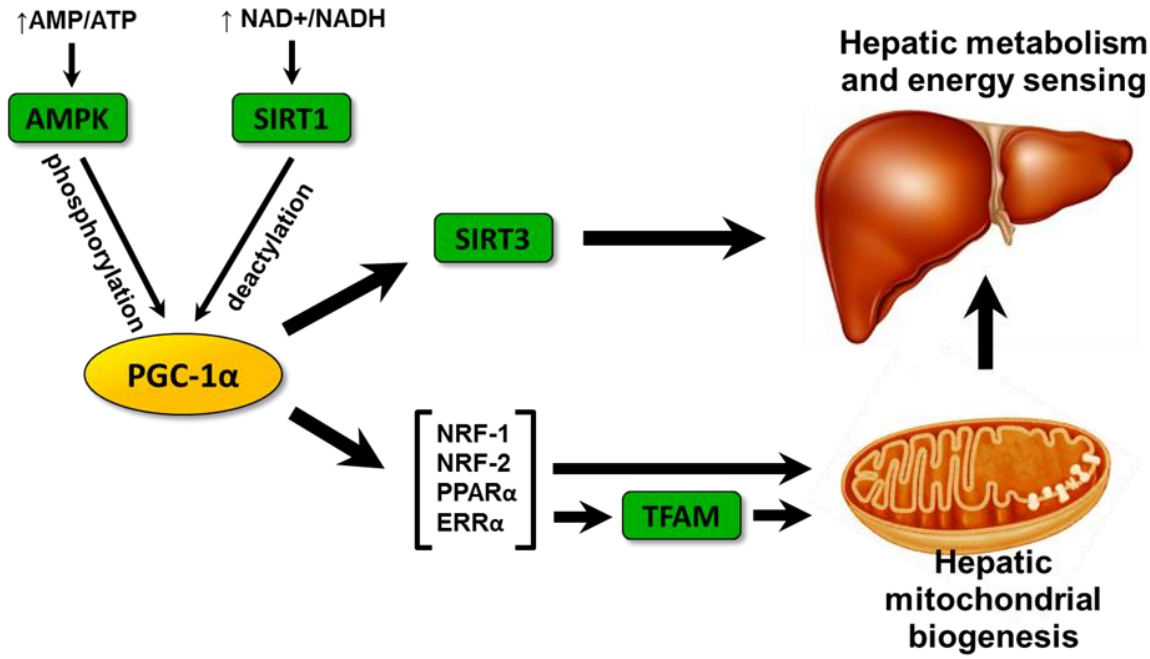

Figure 5 | **PGC1α is central to the regulation of hepatic mitochondrial biogenesis in response to energy signalling pathways**. Decreased intracellular energy levels promote AMPK and SIRT1 activity, which increase PGC1α activity by posttranslational mechanisms. PGC1α upregulates mitochondrial biogenesis via TFAM and other transcription factors.

Metformin is now routinely prescribed to women with gestational diabetes and TIIDM during pregnancy in the UK[303]. Metformin treatment in diabetic pregnancy is not associated with any increased risk of complications or morbidity over insulin treatment[304], and there seems to be little difference between offspring of metformin- or insulin-treated women with gestational diabetes at 2 years of age[239]. Metformin is therefore protective against fetal and neonatal morbidity and mortality associated with maternal diabetes. In pregnant women without gestational diabetes but with obesity, metformin treatment was safe, despite not decreasing the risk of babies being born large for gestational age compared with women receiving placebo[303]. As metformin freely crosses the placenta and accumulates in amniotic fluid[208-210], metformin can exert direct effects on exposed fetal tissues. Despite use in human pregnancy, the effect of metformin on fetal physiology and development is not well-known, particularly in the context of modulation of the persistent changes to the liver associated with maternal obesity.

The effect of metformin on fetal liver lipids and lipid-related gene expression in obese mouse pregnancy has been explored by Harris *et al.* at E19[256]. Showing that maternal obesity or metformin treatment had no effect on total fetal liver TAG, they also found no effect of metformin on a number of genes upregulated in untreated fetuses born to HF dams, including *Fas*

(encoding fatty acid synthase), *Lxra* (encoding liver X receptor alpha) and *Scd1* (encoding stearoyl-Coenzyme A desaturase 1). Upregulation of FAS is associated with increased DNL, which is a major pathway promoting hepatic steatosis in patients with NAFLD; LXRα is a key regulator of lipogenesis in the liver, and increased activation of the protein is also associated with upregulated DNL. Metformin inhibits mTORC1 signalling in liver cells, which decreases levels of SREBP1c and lipogenic gene expression, including *Fas*[212], yet Harris *et al.* found no effect of metformin on SREBP1 protein levels in their model. Thus, metformin does not seem capable of modulating fetal hepatic lipid metabolism *in utero* during obese pregnancy, at least in mice.

As mentioned earlier, maternal obesity is associated with hepatic mitochondrial dysfunction in adult offspring[70], characterized by impaired electron transport chain activity. Metformin has been shown to increase mitochondrial biogenesis in human umbilical vein endothelial cells, via a mechanism dependent on the transcriptional coactivator peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α)[231]. PGC1α is regulated post-translationally by AMPK and SIRT1, key sensors of cellular AMP and NAD^+ respectively. Increases in both metabolites signal a lack of cellular energy, resulting in the adaptive up-regulation of PGC1α activity, and it's downstream effects, notably gluconeogenesis, mitochondrial biogenesis (via increased expression of transcription factor TFAM) and reduction of reactive oxygen species generation[305]. PGC1α also upregulates nuclear respiratory factor 1 (NRF1), which further promotes TFAM expression[306].

SIRT1 is an NAD^+-dependent deacetylase that upregulates expression of metabolic genes in times of metabolic stress to maintain homeostasis. Key targets of SIRT1 include activation of PGC1α via deacetylation (promoting mitochondrial respiration and lipid catabolism) and inhibition of PPARγ and LXRα (inhibiting lipid anabolism), properties that have made enhancing hepatic SIRT1 activity a target for drug development[307]. SIRT1 expression in fetal liver tissue is decreased in NHP and murine models of maternal obesity[308-310], and blockade of SIRT1 in human fetal hepatocytes promotes increased intracellular glucose and lipid levels[311].

Importantly, metformin increases the activity of AMPK and SIRT1 as a result of its inhibition of complex I of the mitochondrial electron transport chain, which results in increased AMP/ATP and NAD+/NADH ratios[274]; thus, metformin may counteract the suppression of these key proteins during development in obese pregnancy. However, the inappropriate activation of SIRT1 and AMPK during lean pregnancy may also be detrimental to the offspring. For instance, activation of SIRT1 suppresses adipocyte formation from mesenchymal stem cells in a mechanism dependent on PPARγ inhibition, which may impair the development of functional adipose tissue[312]. However, studies investigating these effects *in vivo* in fetuses are lacking.

Given the paucity of data on fetal and maternal outcomes in pregnancies (both lean and obese) treated with metformin, we investigated the short-term acute impacts of both maternal obesity and metformin treatment in a mouse model, and characterized the expression of key fetal hepatic metabolic genes previously shown to be modulated by maternal HF diet and/or obesity, or metformin: *Sirt1, Sirt3, Pgc1a, Nrf1* and *Tfam*.

4.2 Methods

4.2.1 Animal model

As described in Chapter 3, female C57BL/6 mice were assigned to either a high-fat diet (HF; 45% kcal fat, 20% kcal protein, 35% kcal carbohydrate; Special Diet Services, UK) or standard chow diet (C; 7.5% kcal fat, 17.5% kcal protein, 75% kcal carbohydrate; RM1—Special Diet Services, UK), given *ad libitum* for 6 weeks prior to and then during mating and pregnancy, generating two maternal groups: C and HF. Dams were weighed every 2 weeks and were subjected to a 2-hour intraperitoneal glucose tolerance test prior to mating (IPGTT).

4.2.1.1 Assessment of glucose tolerance in pregravid females

Before mating, dams were fasted overnight before undergoing an IPGTT, as described in Chapter 3.

4.2.1.2 Mating and metformin treatment

C and HF-fed dams were mated with C-fed C57BL/6 males, with males removed when mating was confirmed by vaginal plug presence. 250mg/kg/day of metformin (Sigma-Aldrich, UK), corresponding to a concentration of 1.75mg/ml, was administered orally in drinking water *ad libitum* to a subset of HF (HFm) or C (Cm) fed dams from plug confirmation until culling at gestational day 16 (E16.5), generating four maternal groups: C, Cm, HF and HFm.

4.2.1.3 Assessment of glucose tolerance in a subgroup of pregnant females

Fasted dams (C, n=4; Cm, n=4; HF, n=4; and HFm = 4) were subjected to a 2-hour IPGTT (see Chapter 3) to assess the acute impacts of metformin treatment and obesogenic HF diet on glucose tolerance and fasted blood glucose concentration. After completion of the IPGTT, dams were killed between 10-11am by cervical dislocation. As fasting can adversely affect fetal development and gene/protein expression to varying amounts depending on maternal fuel reserves, fetal tissues for biochemical analysis related to these studies were not obtained but were stored for subsequent analysis.

4.2.1.4 Metformin quantification

Metformin was quantified in dam blood plasma and amniotic fluid by laser diode thermal desorption/atmospheric pressure chemical ionisation tandem mass spectrometry as outlined in Chapter 3.

4.2.1.5 Sampling of fetal and placental tissue at E16.5

Unfasted pregnant dams from each treatment and dietary group (C, n=5; Cm, n=3; HF, n=9; and HFm, n=7) were killed at 10-11am on E16.5 by anaesthetising with isoflurane and exsanguinating via cardiac puncture, followed by cervical dislocation. Fetuses were excised and killed by decapitation, and liver, brains and hearts removed. Maternal and fetal tissue was quickly collected and snap frozen in liquid nitrogen before storage at -80°c. Maternal and fetal blood was assayed for glucose concentration by handheld glucometer (Roche Diagnostics), before maternal blood was collected in heparinized tubes and centrifuged and aliquoted plasma stored at −20°C. Maternal bodyweight (pre-killing), heart and left liver lobe weights, litter size, and weights of fetuses and fetal livers were recorded. Molecular analysis of fetal tissues was conducted using methods described below.

4.2.1.6 Analysis of fetal hepatic metabolic gene expression

Total RNA was extracted from fetal liver by methods outlined in Chapter 3. Briefly, tissue was homogenised in Tri reagent (Sigma-Aldrich), and isolated according to the manufacturers instructions. Concentration and purity of extracted RNA samples was assessed by NanoDrop Spectrophotometer (NanoDrop 1000, Thermo Scientific, USA).

cDNA synthesis was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT; Promega, UK) with random primers using 1000ng of extracted RNA template. Real-time qPCR was then performed using pre-optimised gene of interest TAQMAN probes, primers and reagents (all Eurogentec, UK) against *Sirt1, Sirt3, Nrf1, Pgc1a* and *Tfam*. PCRs were run on an ABI7500 real-time PCR system (Applied Biosystems, USA). The housekeeping gene *Ywhaz* was used to normalise gene of interest data using the ΔΔCt method[282].

4.2.1.7 Statistics

As described in Chapter 2, t-tests were used to test differences when only two groups were assessed (pre-mating C and HF dam parameters). Data reported from these comparisons are observed data.

Mixed models were used to quantify estimated fixed effect size and significance for the three factors present (maternal HF diet, maternal metformin treatment, and the HF diet*metformin interaction term) in studies of dams and fetuses at E16.5. $P < 0.05$ was considered significant. Graphed data are predicted values derived from maximal models (containing all 3 factors and litter size).

Data are reported as the estimated fixed effect size (FE), which represents the estimated effect of that independent variable (for instance, maternal HF diet) on the dependent variable (for instance, maternal pregravid bodyweight). Standard errors and P values for the effect are also reported. Dependent variables that were not normally distributed on visual inspection and assessment of skew and kurtosis were log transformed (as described in Chapter 2). As modelled log-transformed data cannot be meaningfully back-transformed (owing to the non-linear nature of the transformation, logFEs and associated standard errors and P values are reported for these variables.

Tables accompany each figure to summarise the statistically significant fixed effects on each respective dependent variable.

4.3 Results

4.3.1 High-fat diet induces obesity in female mice

Dams fed a HF diet for 6 weeks prior to mating had a higher pregravid bodyweight than dams fed a C diet (37.8 ± 1.05g vs 27.1 ± 0.66g, P < 0.0001) (Figure 6). HF dams also showed impaired pregravid glucose tolerance as assessed by IPGTT (AUC 2128 ± 132mmol/l/min vs 1398 ± 32.59 mmol/l/min, P < 0.001), and had higher fasting blood glucose (7.45 ± 0.16 mmol/l vs 5.57 ± 0.23 mmol/l, P < 0.0001) than C dams.

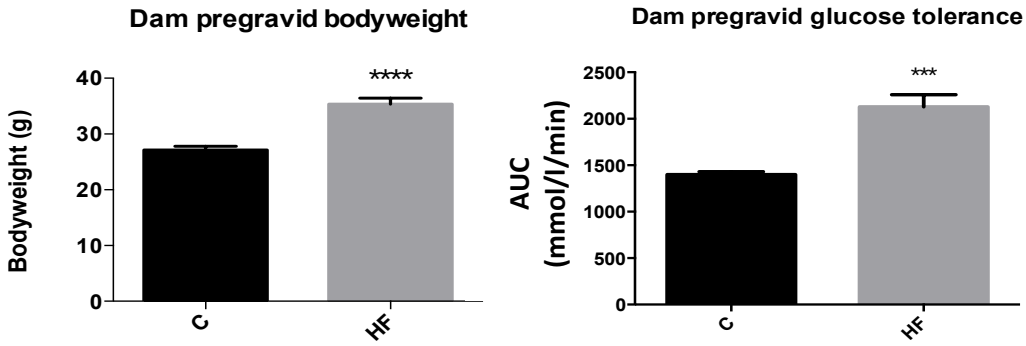

Figure 6 | **Dam pregravid bodyweight and glucose intolerance is increased by feeding an HF diet.** After 5-6 weeks of feeding either HF diet or C diet, females fed HF diet are significantly heavier and exhibit significantly greater glucose. C, n = 7; HF, n = 7. ***, P < 0.001; ****, P < 0.0001 vs C. C, chow diet; HF, high-fat diet.

4.3.2 Metformin dissolved in drinking water produces clinically relevant plasma and amniotic fluid concentrations

Non-fasted plasma and amniotic fluid from dams given metformin (Cm and HFm dams) at time of death (E16.5) was sent to collaborators at AstraZeneca (Cambridge, UK) for measurement of metformin concentration to confirm appropriateness of the dosage. Plasma metformin concentration was 1226 ± 439ng/ml in Cm dams (n = 3) and 857 ± 269ng/ml in HFm dams (n = 7); there was no significant difference between the groups. There was also no difference in amniotic fluid concentration, which was 919 ± 182ng/ml in Cm dams (n = 3) compared with 978 ± 202ng/ml in HFm dams (n = 4). Metformin was below detectable concentrations (<2.8ng/ml) in plasma and amniotic fluid samples from untreated C and HF dams.

4.3.3 Effect of maternal diet and metformin treatment on dams at E16.5

Body weight of dams (before excising fetuses, placentas and amniotic fluid) at E16.5, prior to death, was assessed in the non-fasted E16.5 dam cohort (Figure 7, Table 6). HF diet significantly increased dam body weight (fixed effect (FE) 5.93 ± 2.40g, P = 0.022), while metformin did not independently alter body weight. Litter size in these dams was reduced by HF diet (FE -3.07 ± 0.63 pups, P < 0.0001). There was a significant interaction between maternal diet and metformin treatment (HF diet x metformin FE 2.90 ± 0.93 pups, P < 0.05) due to decreased litter size with metformin treatment in Cm vs C dams, but increased litter size with metformin treatment in HFm dams vs HF dams.

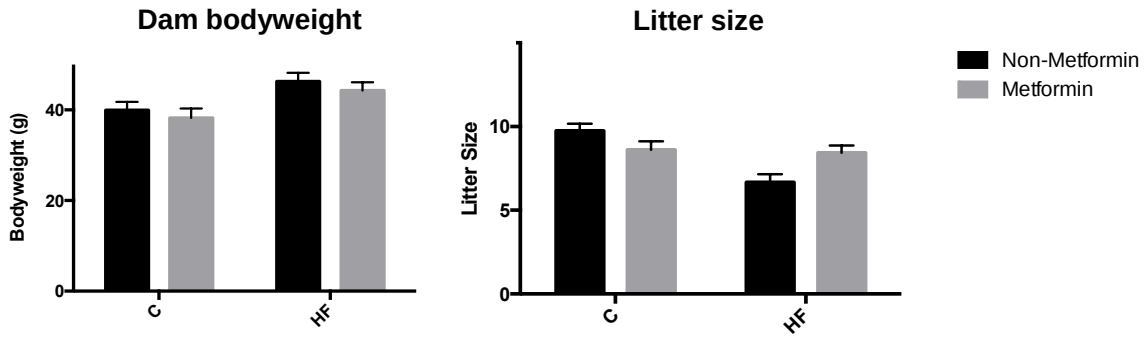

Figure 7 | **Dam body weight and litter size.** Dam bodyweight at E16.5 (left panel). Litter size is reported in non-fasted dams at E16.5 (right panel). Data is presented as observed means ± SEM. Significant effects of diet, treatment and interactions are described in main text. C, n = 5; Cm, n = 3; HF, n = 9; and HFm, n = 7. C, chow; HF, high fat.

Dependent variable	Significant effects	Fixed effect est.	P
Dam body weight	Maternal HF diet	5.93 ± 2.40 g	= 0.022
Litter size	Maternal HF diet	-3.07 ± 0.63 pups	< 0.001
	Maternal HF diet * metformin	2.90 +- 0.93 pups	< 0.05

Table 6 | Statistical results for Figure 7.

Although non-fasted measurement of metabolic parameters altered by feeding is not performed clinically due to variation resulting from timing of last feeding, plasma levels of glucose, insulin, cholesterol and TAG were assessed (Figure 8, Table 7). Non-fasted plasma glucose level was increased by HF diet (HF diet FE 1.96 $\pm$ 0.80 mmol/l, P = 0.02), although metformin treatment had no significant effect, and there was no interaction between treatment and maternal diet. Non-fasted plasma insulin concentration was significantly increased as a result of HF diet (HF diet logFE 0.49 $\pm$ 0.14, P = 0.003). There was a significant interaction between metformin and HF diet, driven by the marked decrease in insulin levels as a result of metformin treatment in HF dams (maternal diet x metformin interaction logFE -0.42 $\pm$ 0.19, P = 0.044). There was no effect of either diet or metformin treatment on non-fasted insulin/glucose ratio, a proxy measure of insulin sensitivity (data not shown). Non-fasted plasma total cholesterol concentration was markedly increased in HF dams (HF diet FE 0.99 $\pm$ 0.26mmol/l, P = 0.002), and there was no independent effect of metformin on cholesterol levels. Plasma TAG levels were unaltered by diet or metformin treatment.

Plasma CRP concentration, a marker of systemic inflammation, was increased by maternal HF diet (HF diet logFE 0.18 ± 0.05, P = 0.004) (Figure 8, Table 7). There was a trend towards a significant interaction effect due to decreased CRP levels in HFm vs HF dams yet increased levels in Cm vs C dams (maternal diet x metformin interaction, P = 0.062).

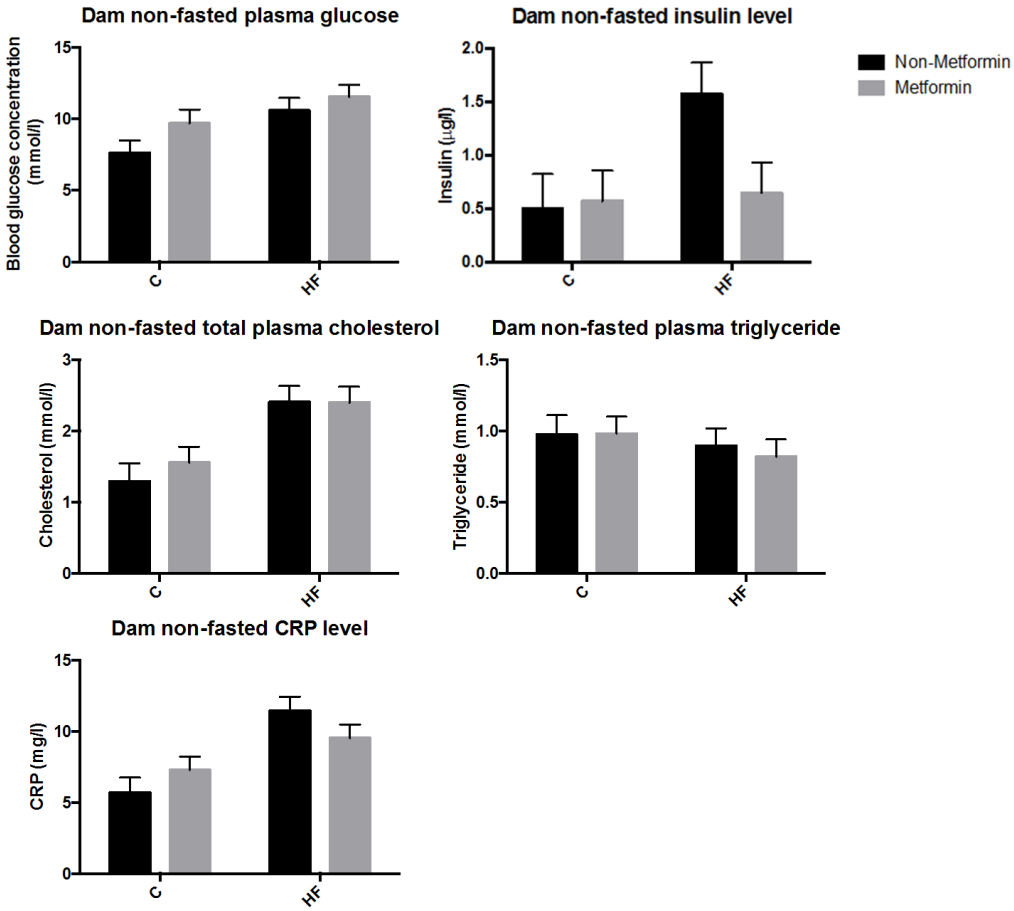

Figure 8 | **Non-fasted dam plasma metabolites and CRP at E16.5.** Data is presented as observed means ± SEM. No pairwise comparisons between treated and untreated dams on the same diet were significant. Significant effects of diet, treatment and interactions are described in main text. C, n = 5; Cm, n = 3; HF, n = 9; and HFm, n = 7. C, chow; HF, high fat.

Dependent variable	Significant effects	Fixed effect est.	P
Non-fasted plasma glucose	Maternal HF diet	1.96 ± 0.80 mmol/l	= 0.02
Non-fasted plasma insulin	Maternal HF diet Maternal HF diet * metformin	0.49 ± 0.14 log µg/l 0.42 ± 0.19 log µg/l	= 0.003 = 0.044
Non-fasted TC	Maternal HF diet	0.99 ± 0.26 mmol/l	= 0.002
Non-fasted plasma TAG	NONE	NA	NA
Non-fasted plasma CRP	Maternal HF diet	0.18 ± 0.05 log mg/l	= 0.004

Table 7 | Statistical results for Figure 8. HF, high-fat; TAG, triglyceride; TC, total cholesterol

4.3.4 Glucose tolerance in fasted dams at E16.5

Blood glucose concentration and glucose tolerance was measured in a fasted cohort of dams to minimise feeding-induced variation (Figure 9, Table 8). Fasting blood glucose was increased by maternal HF diet (HF diet FE 2.7 ± 1.0 mmol/l, P = 0.018), and was unaffected by metformin

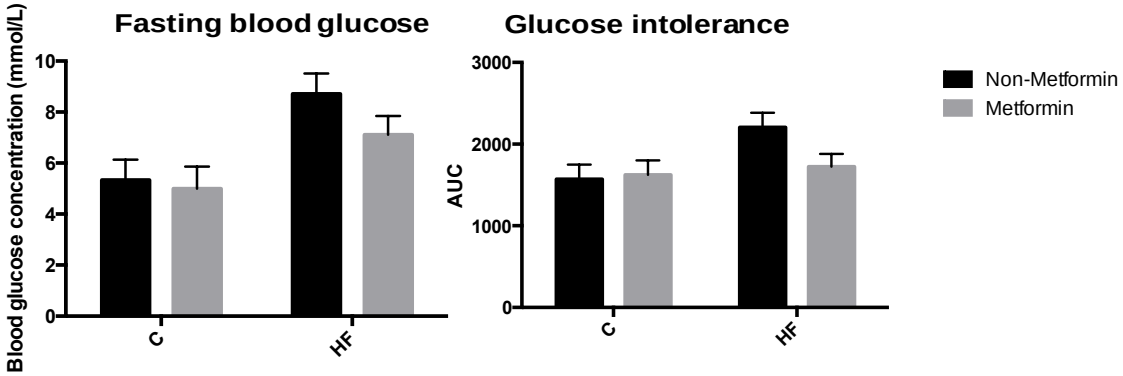

Figure 9 | **Measures of glucose homeostasis in fasting dams at E16.5.** Fasting blood glucose in dams at E16.5 (left panel). Glucose intolerance as assessed by IPGTT (right panel). Data is presented as observed means ± SEM. Significant effects of diet, treatment and interactions are described in main text. C, n=4; Cm, n=4; HF, n=4; and HFm = 4. C, chow; HF, high fat.

treatment. HF diet increased the AUC of glucose disposal (HF diet FE 606 ± 276 AUC, P = 0.047) whereas metformin had no effect.

Dependent variable	Significant effects	Fixed effect est.	P
FBG	Maternal HF diet	2.7 ± 1.0 mmol/l	= 0.018
IPGTT AUC	Maternal HF diet	606 ± 276 mmol/l/min	= 0.047

Table 8 | Statistical results for Figure 9. AUC, area under the curve; FBG, fasting blood glucose; IPGTT, intraperitoneal glucose tolerance test.

4.3.5 Effect of metformin and maternal diet on fetal parameters

Fetal weight and the weights of placenta and fetal livers were assessed and recorded in fetuses from non-fasted dams (Figure 10, Table 9). Fetal body weight was decreased by HF diet (HF diet FE -0.12 ± 0.05g, P = 0.018) and by metformin treatment (metformin FE -0.13 ± 0.04g, P = 0.004). There was no interaction between diet and metformin treatment, as metformin lowered fetal bodyweight in both Cm and HFm fetuses. Non-fasted fetal blood glucose was increased by HF diet (HF diet FE 1.49 ± 0.42 mmol/l, P = 0.002) and by metformin in both Cm and HFm fetuses (metformin FE 1.37 ± 0.37 mmol/l, P = 0.001). Fetal liver weight was unaffected by HF diet, but was decreased by metformin treatment (metformin FE -0.01 ± 0.002g, P < 0.0001) (Figure 10). This finding disappeared when liver weight was adjusted for fetal bodyweight. Placenta weight

was unaltered by maternal diet or metformin treatment. However, when expressed as a percentage of fetal weight, HF diet (HF diet FE 5.6 ± 2.2%, P = 0.019) and metformin treatment (metformin FE 4.6 ± 1.9%, P = 0.024) increased placental weight; thus, this decreased placenta:fetus ratio is dependent on altered fetal weight (data not shown).

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Fetal body weight	Maternal HF diet Metformin	-0.12 ± 0.05 g -0.13 ± 0.04 g	= 0.018 = 0.004
Non-fasted fetal blood glucose	Maternal HF diet Metformin	1.49 ± 0.42 mmol/l 1.37 ± 0.37 mmol/l	= 0.002 = 0.001
Raw fetal liver weight	Metformin	-0.01 ± 0.002 g	< 0.0001
Placental weight (% fetal body weight)	Maternal HF diet Metformin	5.6 ± 2.2% points 4.6 ± 1.9% points	= 0.019 = 0.024

Table 9 | Statistical results for Figure 10. HF, high-fat.

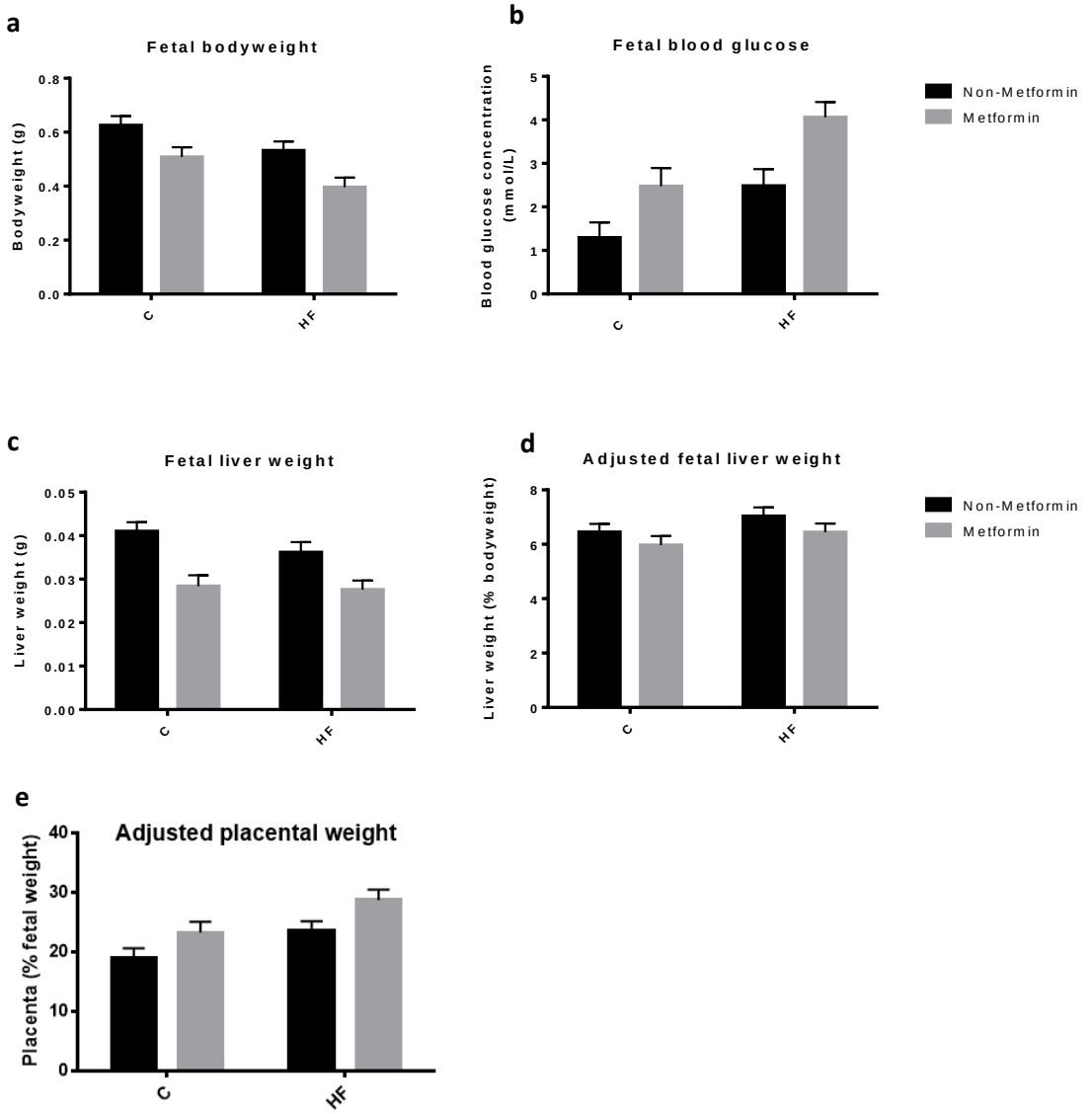

Figure 10 **| Fetal parameters at E16.5. a**. Fetal bodyweight at E16.5. **b**. Non-fasted fetal blood glucose at E16.5. **c**. Unadjusted fetal liver weight. **d**. Fetal liver weight adjusted for fetal bodyweight. **e**. Placental weight adjusted for fetal weight. Data is presented as predicted means ± SEM from maximal models (that is, containing all three fixed effects). Significant main effects and interactions are discussed in the main text. Number of litters: C, n=5; Cm, n=3; HF, n=9; and HFm, n=7. All fetuses from each litter included in analyses (n = 6-11 per litter). C, chow; HF, high-fat.

4.3.6 Metformin treatment and diet alter gene expression in fetal livers

Because acute metformin administration has previously been shown to alter expression of genes regulating mitochondrial biogenesis in cell lines[231,313], and as mitochondrial function has previously shown to be altered by exposure to maternal obesity in rodent models[70,72], we investigated whether hepatic expression of these genes was changed in our model (Figure 11, Table 10). *Pgc1a*, *Nrf1* and *Sirt3* expression was not significantly altered either HF diet or

metformin treatment. mRNA expression of *Sirt1*, a gene known to be positively regulated by metformin[222,314], was increased by metformin treatment (metformin logFE 0.21 ± 0.06, P = 0.004) but not by HF diet. Expression of *Tfam*, a key transcriptional regulator of mtDNA synthesis[315], was increased by metformin treatment (metformin logFE 0.29 ± 0.13, P = 0.05), whereas HF diet had no effect.

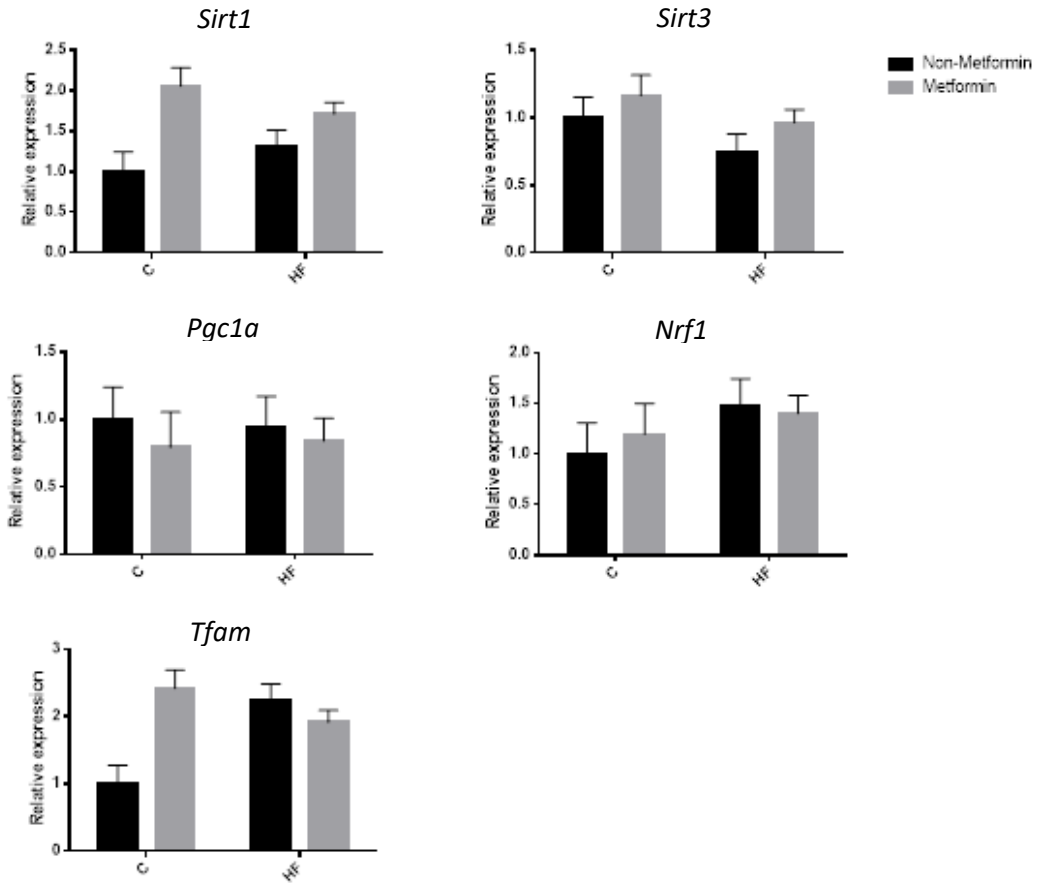

Figure 11 | **Hepatic expression of genes related to energy metabolism in non-fasted fetuses at E16.5**. Data are relative to C/C group and are graphed non-logged, although statistical analysis was performed on logged data. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. Number of litters: C, n=4; Cm, n=3; HF, n=4; and HFm, n=7. Liver from 1-2 fetuses per litter were analysed. C, chow; HF, high-fat.

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Pgc1a hepatic expression	NONE	NA	NA
Sirt3 hepatic expression	NONE	NA	NA
Nrf1 hepatic expression	NONE	NA	NA
Sirt1 hepatic expression	Metformin	0.21 ± 0.06 log AU	= 0.004
Tfam hepatic expression	Metformin	0.29 ± 0.13 log AU	= 0.05

Table 10 | Statistical results for Figure 11. AU, arbitrary units; NA, not applicable.

4.4 Discussion

Although metformin is currently recommended by NICE in the UK for the management of GDM when exercise and diet changes are ineffective[316], limited data exists on the acute effects of metformin on fetal parameters *in utero*, particularly outcomes in the liver, a primary site of metformin action. The data reported here show that, in a well-characterized mouse model of maternal obesity and glucose intolerance during pregnancy, metformin increases fetal hepatic mRNA expression of the key energy-sensitive gene *Sirt1*, and *Tfam*, a primary regulator of mtDNA synthesis. Notably, metformin seemed to reduce fetal bodyweight independently of maternal diet. Together, these data suggest that metformin exerts acute effects on the fetus, and that these changes can be independent of maternal glucose tolerance (used to define GDM in the UK) and adiposity.

Dams fed a HF diet for 6 weeks exhibit glucose intolerance and weight gain, in line with previous reports using this model[70,174], and confirming the presence of an obese pregnancy phenotype. Analysis of plasma and amniotic fluid from dams given metformin verified the presence of metformin at human therapeutic concentrations[317], validating the dosing method used in this study.

At E16.5, metformin had no effect on maternal body weight, mirroring data from human studies[243]. There was also no significant effect of metformin on fasting blood glucose or glucose tolerance, a finding that might be due to the interactive effect of HF diet and pregnancy-induced insulin resistance masking glucose-sensitizing effects of the drug. In addition, fasted insulin, which was not measured in these dams, is often elevated in the absence of overtly elevated fasting blood glucose in humans [318] and mouse models of obesity, including adult offspring in this model[269]. As such, the beneficial effects of metformin during pregnancy might be observed in reduced fasting insulin concentrations. Non-fasting, random sampled insulin was lower in HFm dams than HF dams (0.64 vs 1.57 ng/ul), resulting in a significant interaction between maternal HF diet and metformin treatment and suggesting improvement in insulin sensitivity in HFm dams, yet this finding needs to be confirmed with fasting measurement of plasma insulin levels. Interestingly, the EMPOWaR study found a lack of effect of metformin on human maternal glucose homeostasis parameters, including HOMA-IR and 2 h glucose concentration, at 36 weeks gestation, although effects of metformin were observed at 28 weeks gestation[243]. These findings support a role for pregnancy-related insulin resistance, which increases during later gestation stages[101], masking the glucose-lowering efficacy of metformin, and might explain why no effect on glucose tolerance was observed in data reported in this thesis. There was a trend towards metformin reducing plasma CRP concentrations in the model reported here, a finding also

reported in the EMPOWaR study. CRP is a well-defined marker of systemic inflammation, is raised in obesity states and is associated with the degree of glucose intolerance[319]. Levels of plasma CRP are also associated with preeclampsia risk in women, and infusions of CRP induce preeclampsia features in a mouse model of the condition[320]. Together, these findings and those presented here suggest that metformin may have beneficial short-term effects in mothers independent of its metabolic actions.

Fetal bodyweight at E16.5 was decreased by maternal HF diet and metformin treatment. Although human fetuses exposed to maternal obesity *in utero* are typically large for gestational age, risk of fetal growth restriction is increased in pregnancies complicated by obesity[321]. Fetal growth restriction is also observed in some animal models of maternal obesity, although the exact effects differ depending on the species, strain, diet, gestational time point and experimental conditions employed[163,322,323]. In studies observing multiple fetal time-points, catch-up growth has been shown; Sferruzi-Perri *et al.* found impaired fetal growth in a rat model of maternal obesity at E16, but by E19 exposed fetuses were the same weight as non-exposed fetuses, despite altered placental morphology[162]. Notably, fetuses exposed to metformin in lean dams also suffered growth restriction. This finding mirrors results from Salomaki *et al.*, who showed that metformin at similar concentrations to those used in this study reduced fetal bodyweight in a mouse model at E18.5. Together, these data prompt further exploration of the acute effects of metformin in lean pregnancies.

Impaired placental development and consequent altered utero-placental blood flow has been advanced as a mechanism common to both fetal overgrowth and fetal undergrowth in response to maternal obesity[324], although this pathway was not assessed in this study. The finding of increased placental weight relative to fetal bodyweight, adjusted for litter size, in animals exposed to maternal HF diet or metformin (independent of maternal diet) could represent an adaptive response to insufficient placental nutrient transport[325,326]. For instance, in humans, small and efficient placentas seem to convey the lowest risk of cardiovascular disease programming in adulthood[327]. Specific data on the effect of metformin on placental efficiency are lacking, and these novel initial findings in the context of maternal obesity suggest future work should assess whether metformin, independent of maternal parameters, alters placental physiology.

Interestingly, metformin protected HFm dams from loss of pups. As a result, this increased litter size might manifest in reduced fetal weight, owing to distribution of maternal resources amongst a larger number of fetuses. However, litter size was not significantly predictive of fetal bodyweight when modelling the effects of maternal diet and metformin treatment. The mechanisms by which metformin might increase litter size are unclear. Given that metformin was

administered from confirmation of plug (E0.5), it may produce beneficial effects on blastocyst implantation, blastocyst viability, placental growth or fetal resorption early in pregnancy. However, data on mechanisms that might underpin these effects are lacking. The demonstration that metformin reduces maternal CRP might be indicative of broader anti-inflammatory effects at the level of the endometrium, improving blastocyst implantation and pregnancy success. In addition, Eng *et al.* showed that, in mouse embryos exposed to high levels of IGF1 (which are raised in obesity), metformin activated AMPK in the blastocyst, increasing implantation success[328].

Metformin is known to acutely regulate gene expression through a number of mechanisms, notably activation of AMPK, which orchestrates a catabolic response, including stimulating mitochondrial biogenesis[315]. Moreover, previous work has identified altered mitochondrial function in offspring of obese dams, including in the liver[70,72,182,261]. As a result, mRNA expression of key regulators of mitochondrial biogenesis were quantified in hepatic tissue by RT-qPCR. Hepatic levels of *Tfam* were increased as a result of metformin treatment, although the main effect did not quite reach significance (P = 0.05) due to high between-litter variance. *Nrf1*, which can also directly promote mitochondrial biogenesis, was unaffected by metformin treatment or diet. Limited evidence suggests that metformin can directly increase mitochondrial biogenesis in a human adipocyte cell line via increased *NRF1* and *TFAM* expression, but the characterization of the effects of metformin on these genes in fetal liver in the context of maternal obesity has not performed before.

Hepatic expression of *Sirt1*, a deacetylase that is sensitive to the cellular NAD^+/NADH ratio and likely increases mitochondrial biogenesis, was not found to be altered by maternal HF diet, in contrast to previous studies in developmental priming models[308,309]. However, metformin treatment increased *Sirt1* expression independently of maternal diet. In the context of developmental priming of offspring NAFLD by maternal obesity, this novel finding may be clinically important, as SIRT1 also inhibits SREBP1c, a master driver of lipogenic pathways, via deacetylation[329]. Fetal hepatic SREBP1c has been shown to be upregulated in animal models of maternal obesity, and is associated with increased fetal steatosis[330]. Loss of *SIRT1* expression in human fetal hepatocytes leads to accumulation of glucose and lipids as a result of derepression of gluconeogenesis and lipogenesis, suggesting a protective effect of *Sirt1* against upregulation of these processes and therefore against early-life priming of NAFLD-associated mechanisms[331]. It remains to be seen whether changes to *Sirt1* expression seen in this study or others are persistent in adult life, or whether acute changes in fetal hepatic *Sirt1* levels directly alter adult risk of cardiometabolic disease.

The marked increase of *Sirt1* expression in Cm fetuses is a potentially worrying finding, given that inappropriately high *Sirt1* activity results in a phenotype in mice that mimics calorie restriction[332]. Reduced fetal weight in Cm offspring also lends support to this hypothesis, as reduced fetal size is indicative of maternal conservation of resources[333]. As calorie restriction during pregnancy is also associated with the programming of offspring metabolic disease, including NAFLD[334,335], in later life, this response may be detrimental for long-term offspring health. To conclude this chapter, maternal obesity and glucose intolerance induced by HF diet feeding results in fetal growth restriction, reduced litter size and reduced markers of placental efficiency at E16.5. In dams, metformin treatment does not restore glucose sensitivity or alter bodyweight, although plasma CRP concentration was reduced. Metformin treatment restores litter size in offspring of HF dams, yet otherwise fails to improve placental and fetal phenotypes. Maternal metformin treatment significantly altered hepatic expression of *Sirt1*, and may increase expression of *Tfam*, promoting mitochondrial biogenesis, which is impaired in adult offspring exposed to maternal obesity. However, metformin treatment in lean Cm dams induces effects that, despite not being concerning in isolation, together constitute a worrying constellation of risk factors for later metabolic disease associated with a calorie restriction phenotype. These factors include reduced fetal weight, impaired placental efficiency and overexpression of *Sirt1* relative to the nutritional status of the fetus. As such, these findings urge more detailed investigation of the acute and persistent impacts of metformin on the fetus during pregnancy, in particular the hepatic response to treatment.

Chapter 5: Maternal metformin exposure alters obesity-mediated priming of male and female offspring physiology

5.1 Introduction

Maternal obesity during pregnancy is associated with widespread effects on offspring physiology in adulthood. Although the studies of these effects in humans are largely observational in nature, a wealth of animal research provides mechanisms to support trends found in human populations. Broadly, the effects of maternal obesity on offspring cardiometabolic health can be divided into those that increase risk of obesity and/or its severity (and thereby risks of related comorbidities), and those that directly alter systems related to cardiometabolic health independently of effects on obesity risk, such as insulin sensitivity or adipose tissue cytokine release.

5.1.1 Effects on obesity risk through modulation of energy intake and expenditure

Maternal obesity during pregnancy is associated with increased offspring adiposity, independent of postnatal diet. In humans, exposure to obesity during pregnancy is associated with increased offspring BMI in early childhood[336] and adulthood[337]. These findings are recapitulated in animal models of maternal obesity: rodent, ovine, swine and NHP models have all shown priming of offspring adiposity[130]. The partition of adipose stores between subcutaneous and visceral depots has been associated with risk of metabolic disease. Increased visceral adipose deposition relative to subcutaneous adipose tissue expansion increases hepatic insulin resistance and inflammation and leads to an unfavourable blood lipid profile and raised serum pro-inflammatory cytokine levels[15]. As a result, appropriate subcutaneous adipose tissue expansion can be regarded as a protective buffer against excessive energy intake, acting as a sink to prevent ectopic and visceral fat accumulation[338].

Viewed simplistically, obesity is the result of chronic net energy intake[339]. This state occurs when energy consumed in the diet exceeds the energy expended. Total daily energy expenditure (TDEE) in an organism is the sum of basal metabolic rate (BMR), the energy cost of physical activity, the energy cost of food processing (known as the thermic effect of food) and the energy expended maintaining optimal body temperature (thermogenesis)[340]. Although some studies have suggested that individuals with a low BMR or resting metabolic rate (BMR plus the energetic cost of thermogenesis) are at increased risk of obesity[341], other studies show no increased risk[342]. Lean body mass (or fat-free mass, FFM), but not BMI or fat mass (FM), is strongly associated with meal size and daily energy intake in humans[343], suggesting the existence of FFM-derived signals influencing appetite and maintaining overall energy homeostasis[343]. The single largest component of RMR is the energy cost of maintaining fat-free mass (FFM); ~60-70% of RMR is due to FFM, whereas fat mass (FM), which is relatively metabolically inert, accounts for only 6-7%. Age and gender exert very small significant effects on RMR, while around a quarter of residual RMR

variation remains unexplained, after excluding analytical error[344]. Because of the contribution of FFM, FM, age and gender, RMR varies substantially between individuals[344]. Although the cost of physical activity, whether exercise-based or non-exercise-based, can be a susbtantial contributor to TDEE, evidence suggests that homeostatic mechanisms promote compensatory increases in energy intake through modulation of appetite and satiety signalling[345]. However, these compensatory increases are only seen in longer term (around 2 weeks) experiments, with coupling between energy intake and physical activity level weak over shorter (around 24 h) time frames[346]. Limited evidence suggests that physical activity in adult offspring is reduced as a result of exposure postnatal overnutrition[347,348], yet scant data exists on the priming of activity by *in utero* exposures.

As a result of the uncertain relationship between energy expenditure and obesity risk, research has focused on the modulation of energy intake, which is driven by changes in hunger, satiety and hedonistic reward pathways. Hyperphagia is a common finding in animal models of maternal obesity, which suggests central alterations to pathways regulating food intake[172,349]. Evidence suggests a wealth of hypothalamic changes in offspring as a result of maternal overnutrition, including central leptin resistance, altered neuronal connections between the arcuate nucleus and the paraventricular nucleus and changes to the expression of appetite and satiety related genes[350,351]. In sum, these alterations promote excess energy intake and the development of obesity.

5.1.2 Effects on offspring cardiovascular health

Maternal obesity is associated with impaired cardiovascular function in offspring. In humans, maternal BMI correlates with offspring mortality from CVD[352], and with offspring cardiometabolic risk factors and systolic and diastolic risk factors, in adulthood[296]. An abundance of studies in animal models have demonstrated the programming of cardiovascular function in offspring. Offspring exposed to maternal obesity are hypertensive[174,175], show cardiac hypertrophy[179,353] and have endothelial dysfunction[354].

5.1.3 Sex differences in priming of offspring cardiometabolic function

Priming of offspring cardiometabolic health appears sexually dimorphic in studies reporting results from both male and female offspring. However, the majority of studies only report data on one sex. When male and female offspring are directly compared, evidence of primed metabolic disease by maternal overnutrition is conflicting. In rodent models, Nivoit *et al.*[349] showed increased risk of obesity and insulin resistance in female offspring only; by contrast, Vickers *et*

al.[355] demonstrated increased risk of the same parameters in male offspring only. Female offspring seem to be more susceptible to hypertension primed by maternal obesity and overfeeding[356,357].

In humans, male offspring seem to be at greater risk of developmental priming of obesity than female offspring; males born to obese mothers show greater insulin secretion during OGTT than females born to obese mothers, suggesting priming of more severe insulin resistance in men than in women[358]. Sexual dimorphism of programming has been suggested to be related to an advantage at the species-level of protecting female fetuses, as reproductive success requires the protection of the entire female physiology, whereas male offspring can reproduce with only moderate fitness and the protection of germ cells[258]. The abundant sex differences observed in the studies discussed above indicate a requirement to investigate both male and female offspring in models of maternal obesity, as characterizing only one sex may result in spurious conclusions.

5.1.4 Interventions

Given the evidence of maternal priming of offspring cardiometabolic disease risk, the past few years have seen an increasing number of studies of interventions targeted before and/or during and/or after pregnancy aimed at mitigating the phenomenon[153]. Lifestyle interventions, comprising dietary advice and exercising programmes of varying intensities, have been studied in detail. However, evidence suggests they are ineffective at reducing incidence of large-for-gestational age, the primary outcome in most studies. For example, the UPBEAT study, a multicentre randomized controlled trial of a lifestyle intervention in 1555 pregnant women with obesity found no effect on incidence of gestational diabetes or the number of infants born large-for-gestational-age[189]. Similar findings have also been reported in other trials (reviewed by Catalano and deMouzon[190]). In consequence, pharmaceutical interventions have been suggested, as these can exert direct and instant physiological effects, and can have fewer issues with compliance[190].

Metformin is considered safe for use in pregnancy, and is recommended by NICE in the UK for the management of gestational diabetes if lifestyle interventions are ineffective [316]. Although recently published trial results have suggested that metformin is ineffective at reducing incidence of large-for-gestational-age births in women who are obese[243,244], human studies necessitate many decades of follow-up to assess adult offspring outcomes, leading to the development of animal models. In mice, Tong *et al.* administered metformin to obese mice throughout gestation and lactation [359]. Metformin reduced the adiposity of male offspring exposed to a maternal HF diet, and marginally improved their glucose tolerance. Salomaki and colleagues showed that

metformin treatment from E0.5 to E17.5 in obese dams prevented priming of impaired glucose tolerance in offspring when they were later challenged with an HF diet[255]. These offspring also gained less adipose tissue during the HF challenge if their mothers received metformin; there was little difference in these responses between male and female offspring. In contrast to the beneficial effects of metformin on offspring of obese dams, Salomaki *et al.* also showed that metformin treatment in lean dams primed metabolic dysfunction in offspring when challenged with a postnatal HF diet[253], although this dysfunction was most pronounced in male offspring. Metformin exposure *in utero* primed increased adiposity in adulthood in both sexes as a result of HF diet feeding. However, impaired glucose tolerance was exacerbated only in male offspring, suggesting sexually dimorphic effects.

Although the effects of metformin in pregnancy on adult offspring have been modelled in individual studies, the studies reported in this thesis are the first to assess the effects of metformin in both lean and obese dams on aged (30 week) offspring, in both sexes. The studies of Tong *et al.* and Salomaki *et al.* used adult offspring that were ~81 days and ~119 days respectively, whereas offspring in this study design are ~210 days of age. Age is well-recognized to interact with the maternal priming of offspring metabolic disease, with offspring metabolic dysfunction increasing with age[269]. Aging may be required to expose alterations in metabolic homeostasis in offspring exposed to maternal obesity and/or metformin. Moreover, Tong *et al.* only assessed male offspring, and they did not investigate the effect of metformin in lean pregnancy on adult offspring. Thus, the current literature represents an incomplete picture. Given the relative lack of studies investigating how metformin during pregnancy, whether given to obese or lean mothers, affects offspring physiology during adulthood, we investigated the independent effects of metformin, maternal HF diet and postnatal HF diets, as well as the interactions between these factors. We assessed offspring of both sexes, at a time-point chosen to best expose metabolic dysfunction.

5.2 Methods

5.2.1 Animal model

As previously described in Chapter 3, eight groups of offspring were generated from four maternal groups. Dams were fed either chow (C) or high-fat (HF) diet ad libitum for 6 weeks prior to mating. Metformin (Sigma-Aldrich, UK) was administered in drinking water (at a concentration of 1.75mg/ml) after confirmation of mating (by visualization of copulation plug). This protocol generated four dam groups (C, Cm, HF and HFm). At weaning, all offspring were weaned onto either C or HF diet, generating eight offspring groups, defined by maternal diet/treatment and postnatal diet: C/C, Cm/C, C/HF, Cm/HF, HF/C, HFm/C, HF/HF and HFm/HF. All offspring were subjected to various *in vivo* measurements between 26 and 30 weeks of age. Both male and female data is presented.

5.2.2 Measurement of blood pressure

Blood pressure was measured using the CODA™ tail cuff plethysmography system (Kent Scientific, Connecticut, CT) according to manufacturer's instructions when offspring were 27 weeks of age. Briefly, mice were moved into a quiet, warm room (30 ± 2°C) and acclimatized for at least 30 minutes. Mice were then placed in gentle restraining tubes and secured, before again being left to acclimate for 1 minute. Means and SEMs of blood pressure measurements were calculated from three of five consecutive completed measurements, after discarding the highest and lowest readings.

5.2.3 Characterization of glucose homeostasis

At 29 weeks of age, glucose tolerance was assessed using a 2-hour intraperitoneal glucose tolerance test as described in Chapter 3. Briefly, offspring were fasted overnight, and basal fasting blood glucose was obtained from tail tip blood. Animals were then injected with 20% glucose solution (2g glucose/kg bodyweight), and blood glucose concentrations were recorded at 15, 30, 60 and 120 minutes post injection. Area under the curve of the blood glucose concentration for each animal was calculated using GraphPad Prism 6 (GraphPad Software).

5.2.4 Determination of body composition

At 30 weeks of age, after death was confirmed and fasted blood was taken for plasma analyte assessment, offspring body composition was determined using a Skyscan 1176 *in vivo* micro-tomography CT (μCT) scanner at 35 μm resolution (Bruker Corporation, Belgium). Total volume of

the animal and volumes of fat, soft tissue and bone were calculated as described in detail in Chapter 3.

5.2.5 Collection of tissue samples

After assessment of body composition, the following tissues were removed, weighed and snap frozen in liquid nitrogen: heart (whole heart weighed), liver (whole liver weighed, left lobe frozen only) and body fat (WAT (gonadal, retroperitoneal, perirenal and inguinal fat pads) and iBAT (interscapular brown adipose tissue pad)).

5.2.6 Statistics

The effect of maternal diet, maternal metformin treatment and postnatal diet was assessed by mixed models. Full details of the modelling process are described in Chapter 3. Briefly, all dependent variables were first assessed for normality by visual inspection of Q-Q plots, histograms and skewness and kurtosis, and non-normal variables were log transformed. Maximal models were constructed for each dependent variable, containing all three main effects (maternal diet, maternal metformin treatment and postnatal diet) and all four interaction terms. Models were then simplified by removing non-significant interactions systematically. Litter size was included as a fixed effect, and the litter-of-origin was included as a random effect. Data are graphed as the predicted means and standard errors for each group. Significant main effects (maternal HF diet, maternal metformin treatment and postnatal HF diet) and significant interactions are reported in the text, with the estimated marginal mean for that fixed effect (FE) reported, ± SEM and with the P value for the effect. For example, a significant maternal HF diet effect that increases bodyweight by 2.4 ± 0.1 g averaged over all groups is reported as FE 2.4 ± 0.1g, P = X. Thus, the estimated magnitudes of significant effects can be compared.

Tables accompany each figure to report and summarise the significant fixed effects on each dependent variable.

In addition to models estimating the size of fixed effects, a secondary model was also constructed for each variable to assess whether metformin treatment significantly altered the dependent variable when performing pairwise comparisons of different diet groups; for example, C/C vs Cm/C. This data is reported as planned comparison tests in the main text, and indicated by asterisks on graphs.

5.3 Results

5.3.1 Maternal diet, metformin treatment and postnatal diet alters offspring weight gain

Offspring were weighed before the commencement of metabolic testing (glucose tolerance tests and indirect calorimetry/activity measurements) in order to determine maximal body weight at 28 weeks of age (Figure 12, Table 11). In females, maternal diet (FE 6.86 ± 1.69g, P = 0.001) and postnatal diet (FE 14.22 ± 0.87g, P < 0.0001) independently increased offspring body weight. Metformin treatment significantly reduced body weight in offspring of HF dams only, as indicated by the significant metformin x maternal HF diet interaction term (FE -5.53 ± 2.20g, P = 0.03). Notably, Cm/HF were significantly heavier than C/HF offspring in planned comparison tests (FE 5.26 ± 2.29g, P = 0.033). Body composition was assessed by µCT scan to give volumes (mm^3) of lean body tissue, adipose tissue and bone, and degree of adiposity was confirmed by weighing total and individual adipose tissue depots at death. Maternal HF diet significantly increased body fat percentage as assessed by µCT (FE 7.3 ± 1.6% points, P = 0.001) and by adipose depot mass (FE 4.3 ± 1.1% points, P = 0.001). Postnatal HF diet also increased body fat, to a greater degree than maternal diet (µCT: FE, 18.3 ± 0.9% points, P < 0.0001; adipose depot mass: FE 10.3 ± 0.7% points, P < 0.0001). Offspring of HF mothers given metformin had significantly less percentage adipose tissue (µCT: FE, -7.5 ± 2.1% points, P = 0.004; adipose depot mass: FE, -5.7 ± 1.5% points, P = 0.002), predominantly due to decreased adipose percentage in HFm/C vs HF/C animals, rather than beneficial effects in HFm/HF vs HF/HF mice. In planned comparison tests, C/HF offspring had less adipose than Cm/HF offspring (adipose depot mass: FE -4.3 ± 1.46% points, P = 0.009). Interestingly, unadjusted lean body volume was increased by maternal HF diet (FE 2115 ± 695 mm^3, P = 0.01) and postnatal HF diet (FE 2785 ± 377 mm^3, P < 0.0001), indicating that the increased proportion of adipose in offspring exposed to these diets was even greater than might be assumed if lean body mass was static in response to dietary insults.

Dependent variable	Significant effects	Fixed effect est.	P
Female body weight at 28 weeks	Maternal HF diet	6.86 ± 1.69 g	= 0.001
	Postnatal HF diet	14.22 ± 0.87 g	< 0.0001
	Metfomin * Maternal HF diet	-5.53 ± 2.20 g	= 0.03
Female body fat % (µCT)	Maternal HF diet	7.3 ± 1.6 % points	= 0.001
	Postnatal HF diet	18.3 ± 09 % points	< 0.0001
	Metformin * Maternal HF diet	-7.5 ± 2.1 % points	= 0.004
Female body fat % (adipose depot mass)	Maternal HF diet	4.3 ± 1.1 % points	= 0.001
	Postnatal HF diet	10.3 ± 0.7 % points	< 0.0001
	Metformin * Maternal HF diet	-5.7 ± 1.5 % points	= 0.002
Female lean body volume	Maternal HF diet	2,115 ± 695 mm^3	= 0.01
	Postnatal HF diet	2,785 ± 377 mm^3	< 0.0001
Male body weight at 28 weeks	Postnatal HF diet	4.67 ± 1.84 g	= 0.017

	Maternal HF diet * Postnatal HF diet Metformin * Postnatal HF diet	8.67 ± 2.80 g 5.72 ± 2.46 g	= 0.004 = 0.028
Male body fat % (µCT)	Postnatal HF diet Maternal HF diet * Postnatal HF diet Metformin * Postnatal HF diet	16.2 ± 1.3 % points 7.7 ± 3.3 % points 11.3 ± 2.9 % points	< 0.0001 = 0.026 < 0.001

Table 11 | Statistical results for Figure 12. HF, high-fat.

By contrast, in males (Figure 12, Table 11), maternal HF diet had no independent effect on offspring body weight, and postnatal HF diet increased body weight (FE 4.67 ± 1.84g, P = 0.017). Importantly however, there was a significant interaction between maternal and postnatal HF diets in male offspring, resulting in weight gain beyond the independent, additive contributions of maternal and postnatal HF diets (interaction FE 8.67 ± 2.80g, P = 0.004). There was also an interaction between postnatal HF diet and maternal metformin treatment that increased offspring body weight (FE 5.72 ± 2.46g, P = 0.028), accounted for predominantly by the increased weight in Cm/HF vs C/HF animals. µCT-assessed fat volume proportion was increased by postnatal HF diet (FE 16.2 ± 1.3% points, P < 0.0001) but not by maternal HF diet. Maternal metformin treatment in HF dams did not affect offspring fat volume. Body fat volume percentage was significantly increased when maternal HF diet was followed by a postnatal HF diet (interaction FE 7.7 ± 3.3% points, P = 0.026), beyond the independent effects expected of both diets. Notably, postnatal HF diet in offspring of Cm dams markedly increased body fat, as assessed by µCT (FE 11.3 ± 2.9% points, P < 0.001), a finding almost entirely due to the dramatic increase of body fat percentage in Cm/HF vs C/HF offspring. As in females, the assessment of adiposity percentage by adipose tissue depot weights was very similar to results of the µCT scan, and broad findings are the same.

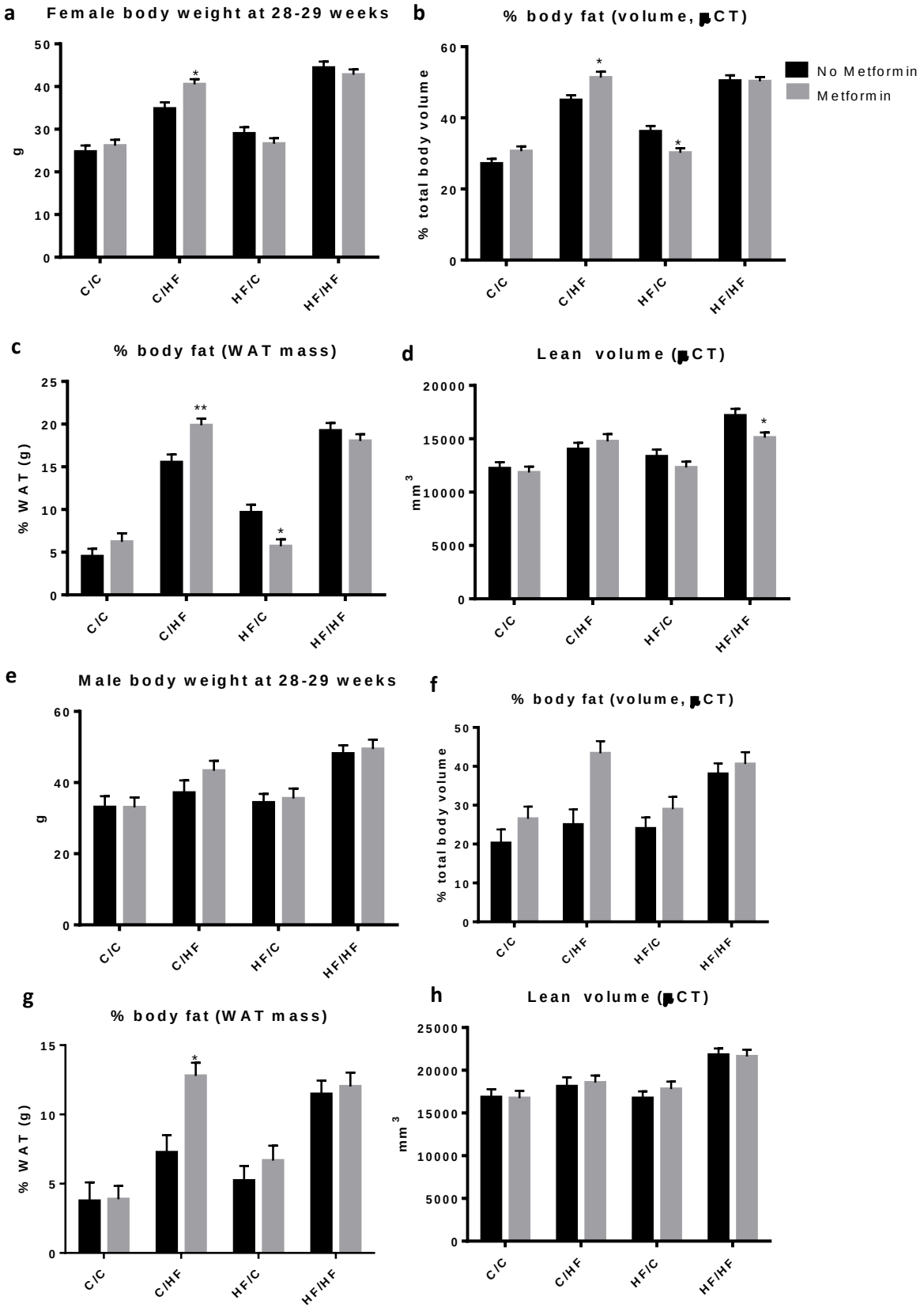

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Figure 12 | **Assessment of adult offspring body composition in females (a-d) and males (e-h). a**. Female offspring body weight. **b**. Female offspring body fat by µCT. **c**. Female offspring body fat by WAT depot mass. **d**. Female offspring total lean body mass by µCT. **e**. Male offspring body weight. **f**. Male offspring body fat by µCT. **g**. Male offspring body fat by WAT depot mass. **h**. Male offspring total lean body mass by µCT. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; vs respective nontreated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat. C, chow; HF, high-fat; WAT, white adipose tissue.

5.3.2 Effect of maternal and postnatal HF diets and metformin on specific adipose tissue depots

Adipose tissue depots were collected and weighed at time of death. These were categorized into subcutaneous adipose depots (inguinal adipose tissue), visceral adipose tissue depots (comprising gonadal, perirenal, retroperitoneal and mesenteric adipose tissue) and brown adipose tissue (scapular depot).

5.3.2.1 Visceral adipose tissue

In females, maternal HF diet (FE 1.58 ± 0.30g, P < 0.0001) and postnatal HF diet (FE 3.10 ± 0.15g, P < 0.0001) both independently increased total visceral adipose tissue mass (Figure 13, Table 12). Metformin treatment in HF dams reduced total visceral adipose tissue (FE -1.58 ± 0.39g, P = 0.002). In planned comparison tests, Cm/HF had greater visceral adipose than C/HF (FE 1.05 ± 0.40g, P = 0.016) whereas HFm/C and HFm/HF offspring had less visceral adipose tissue than HF/C (FE -0.96 ± 0.36g, P = 0.013) and HF/HF offspring (FE 0.98 ± 0.35g, P = 0.008), respectively.

When visceral adipose tissue was expressed as a ratio with inguinal adipose tissue, indicative of the visceral:subcutaneous adipose tissue ratio, maternal HF diet significantly increased the amount of visceral relative to subcutaneous fat (FE 0.3 ± 0.1, P = 0.007). Neither postnatal diet nor metformin treatment affected the ratio.

In males (Figure 13, Table 12), postnatal HF diet significantly increased degree of visceral adipose tissue (FE 0.88 ± 0.34g, P = 0.015), whereas maternal HF diet had no effect. Maternal metformin treatment in offspring given a postnatal HF diet raised visceral adipose tissue (postnatal HF diet x metformin interaction FE 0.94 ± 0.46g, P = 0.048), an effect attributable largely to the increase in Cm/HF vs C/HF offspring.

In contrast to the results in females, the visceral:subcutaneous adipose tissue was decreased in males by postnatal HF diet (FE -1.2 ± 0.3, P < 0.0001), indicating increased deposition of lipid into subcutaneous fat. No other effects were observed.

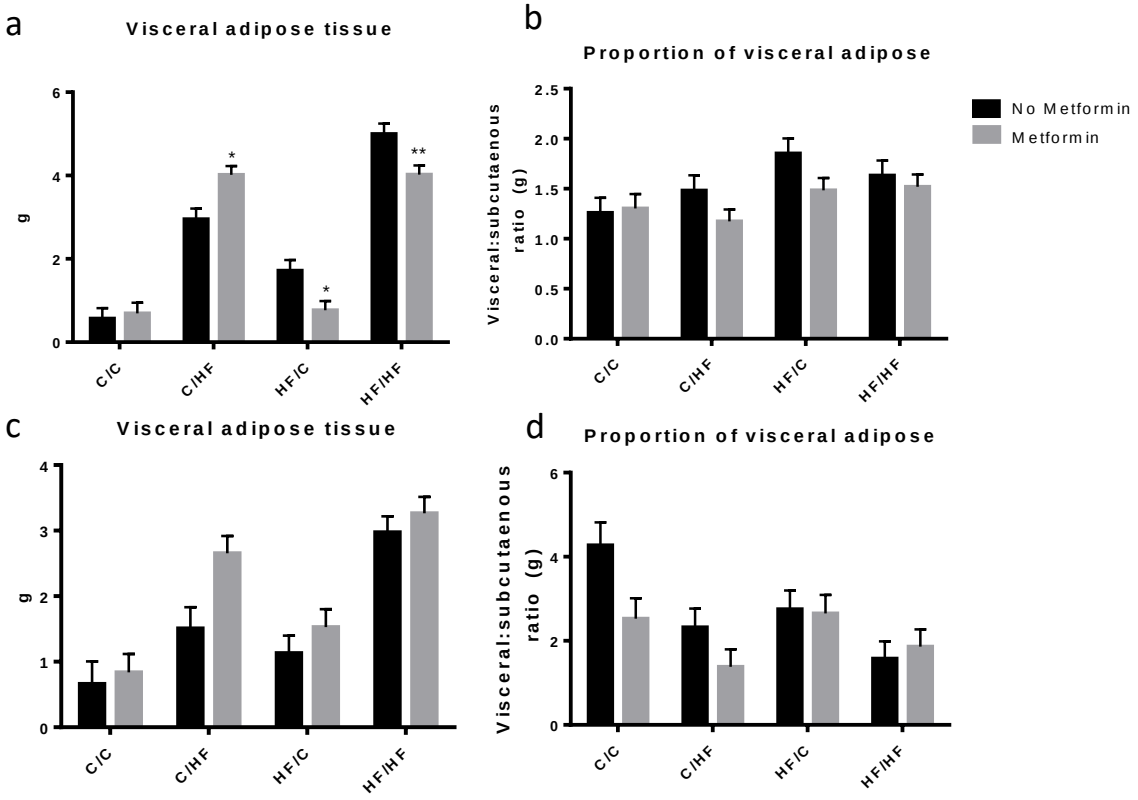

Figure 13 | **Measures of visceral adipose tissue in adult offspring. a**. Female offspring visceral adipose. **b.** Female offspring visceral:subcutaneous adipose ratio. **c**. Male offspring visceral adipose. **d.** Male offspring visceral:subcutaneous adipose ratio. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; vs respective non-treated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat.

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Female visceral adipose tissue mass	Maternal HF diet	1.58 ± 0.30 g	< 0.0001
	Postnatal HF diet	3.10 ± 0.15 g	< 0.0001
	Metformin * Maternal HF diet	-1.58 ± 0.39 g	= 0.002
Female visceral:subcutaneous adipose	Maternal HF diet	0.3 ± 0.1 g/g	= 0.007
Male visceral adipose tissue mass	Postnatal HF diet	0.88 ± 0.34 g	= 0.015
	Metformin * Postnatal HF diet	0.94 ± 0.46 g	= 0.048
Male visceral:subcutaneous adipose	Postnatal HF diet	-1.2 ± 0.3 g/g	< 0.0001

Table 12 | Statistical results for Figure 13. HF, high-fat.

Mass of intrascapular brown adipose tissue (iBAT) in female offspring was not altered by the independent effects of maternal HF diet, postnatal HF diet or maternal metformin treatment (Figure 14, Table 13). There was a significant interaction between maternal and postnatal HF diet, indicating an effect of combined exposure in increasing iBAT mass (FE 0.19 ± 0.05g, P < 0.0001). Moreover, offspring of dams given metformin and receiving a postnatal HF diet had increased amounts of iBAT (postnatal HF diet x metformin interaction FE 0.10 ± 0.04, P = 0.016), attributable to increased iBAT in Cm/HF vs C/HF offspring. There was a significant reduction of iBAT mass in offspring of HF dams given metformin (maternal HF diet x metformin interaction FE -0.16 ± 0.07, P = 0.028), largely resulting from reduced iBAT in HFm/HF vs HF/HF offspring (FE -0.15 ± 0.05, P = 0.006 in planned comparison test).

Data were similar in males (Figure 14, Table 13); iBAT mass was not altered by the independent effects of maternal HF diet, postnatal HF diet or maternal metformin treatment. As in females, combined maternal and postnatal HF diets increased iBAT (maternal HF diet x postnatal HF diet interaction FE 0.29 ± 0.08, P < 0.001), as did maternal metformin in C dams combined with postnatal HF diet (FE 0.21 ± 0.08, P = 0.008). However, metformin in HF dams did not reduce the amount of iBAT in male offspring, in contrast to results in female offspring.

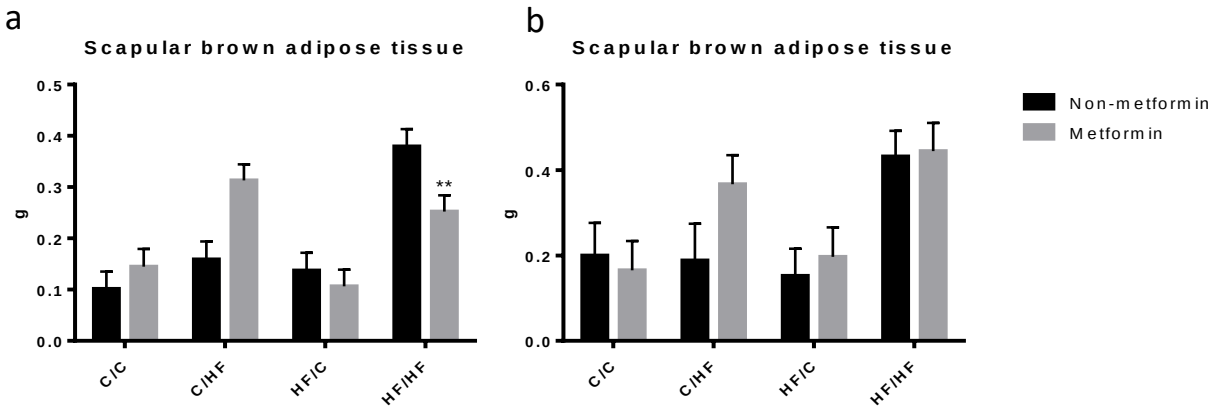

Figure 14 | **Raw mass of iBAT in adult offspring. a**. iBAT mass in females. **b**. iBAT mass in males. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. **, P < 0.01 versus respective untreated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat; iBAT, interscapular brown adipose tissue.

Dependent variable	Significant effects	Fixed effect est.	P
Female iBAT mass	Maternal HF diet * Postnatal HE diet Metformin * Maternal HF diet Metformin * Postnatal HF diet	0.19 ± 0.05 g -0.016 ± 0.07 g 0.10 ± 0.04 g	< 0.0001 = 0.028 = 0.016
Male iBAT mass	Maternal HF diet * Postnatal HE diet Metformin * Postnatal HF diet	0.29 ± 0.08 g 0.21 ± 0.08 g	< 0.001 = 0.008

Table 13 | Statistical results for Figure 14. iBAT, interscapular brown adipose tissue; HF, high-fat.

5.3.3 Offspring blood pressure and heart mass

5.3.3.1 Blood pressure

Systolic and diastolic blood pressure was assessed via tail-cuff plethysmography (Figure 15, Table 14). SBP in female offspring was significantly increased by postnatal (FE 7.8 ± 2.9 mmHg, P = 0.012) but not by maternal HF diet. Interestingly, there was a significant decrease in SBP in offspring exposed to both maternal and postnatal HF diet (FE -9.8 ± 4.6 mmHg, P = 0.04). DBP was also significantly altered by postnatal HF diet (FE 10.1 ± 3.2 mmHg, P = 0.003), yet there was no combined effect of maternal and postnatal HF diets.

In males, no independent variables significantly altered SBP or DBP, demonstrating clear sexual dimorphism.

Dependent variable	Significant effects	Fixed effect est.	P
Female systolic blood pressure	Postnatal HF diet Maternal HF diet * Postnatal HF diet	7.8 ± 2.9 mmHg -9.8 ± 4.6 mmHg	= 0.012 = 0.04
Female diastolic blood pressure	Postnatal HF diet	10.1 ± 3.2 mmHg	= 0.003
Male systolic blood pressure	NONE	NA	NA
Male diastolic blood pressure	NONE	NA	NA
Female heart mass	NONE	NA	NA
Male heart mass	Maternal HF diet Postnatal HF diet	0.019 ± 0.006 g 0.026 ± 0.009 g	= 0.008 = 0.009

Table 14 | Statistical results for Figure 15. HF, high-fat.

5.3.3.2 Heart mass

Heart mass at death was not significantly altered by maternal or postnatal parameters in females (Figure 15, Table 14). In males, conversely, maternal HF diet (FE 0.019 ± 0.006g, P = 0.008) and postnatal HF diet (FE 0.026 ± 0.009g, P = 0.009) both independently increased raw offspring heart mass. In planned comparison tests, HFm/HF male offspring hearts were smaller than HF/HF offspring hearts (FE -0.027 ± 0.012g, P = 0.036).

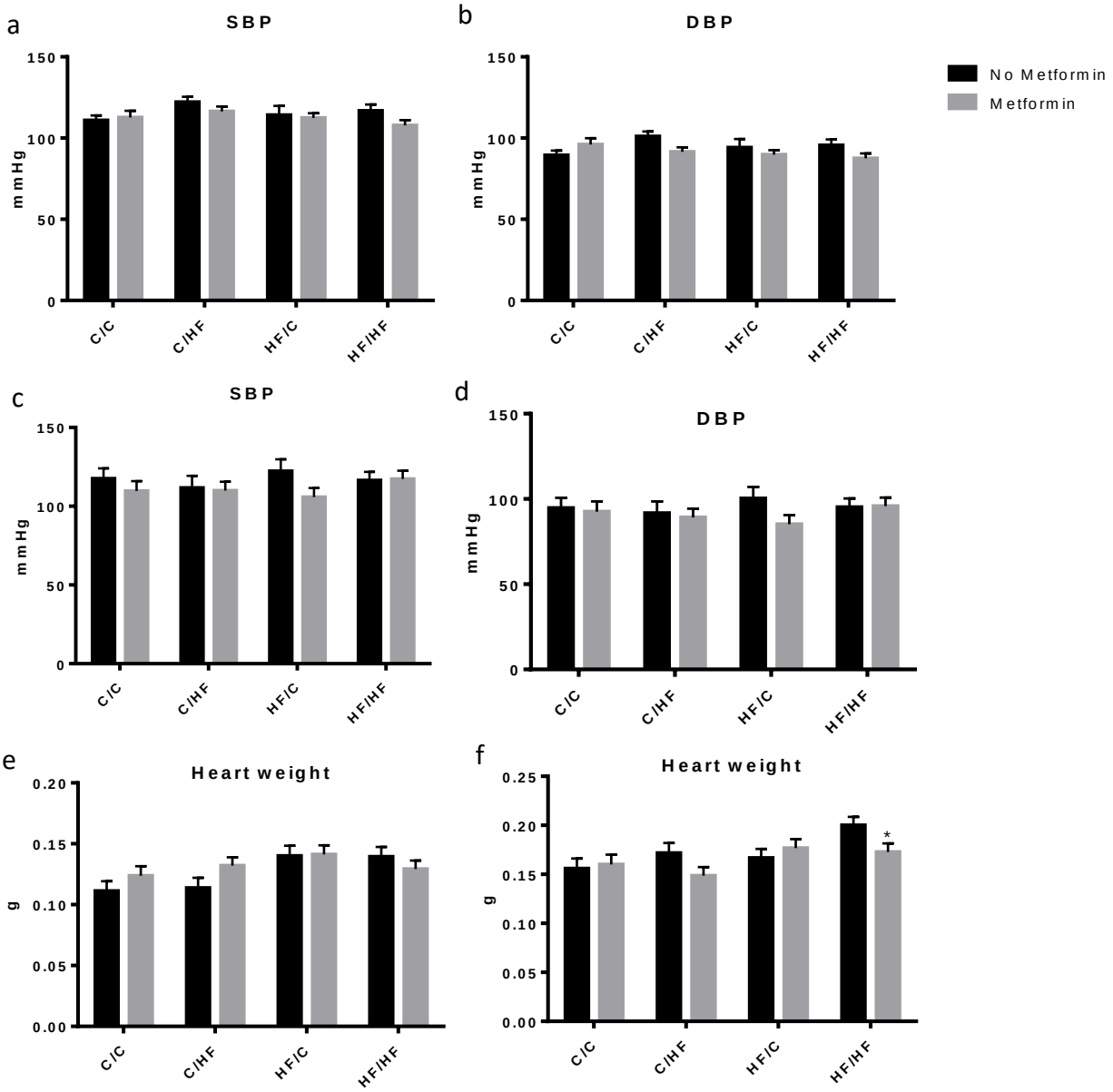

Figure 15 | **Blood pressure and raw heart weight in adult offspring. a.** SBP in female adult offspring. **b**. DBP in female adult offspring**. c.** SBP in male adult offspring. d. DBP in male adult offspring. **e**. heart weight in adult female offspring. **f**. heart weight in adult male offspring. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective non-treated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat.

5.3.4 Offspring glucose homeostasis

Glucose and insulin homeostasis was assessed next in adult offspring (Figure 16, Table 15). Fasting blood glucose (FBG) in females was only significantly increased by postnatal HF diet (FE 1.78 ±

0.48 mmol/l, P < 0.0001). The reduction of FBG in HFm/HF vs HF/HF female offspring was significant in planned comparison testing (FE -2.50 ± 0.97 mmol/l, P = 0.013), but the main effect of metformin treatment was not significant.

In males, both maternal (FE 2.50 ± 0.68 mmol/l, P < 0.001) and postnatal (FE 2.95 ± 0.64 mmol/l, P < 0.0001) HF diets independently increased offspring FBG.

Glucose tolerance in females (assessed by glucose disposal during IPGTT) was decreased by both maternal HF diet (FE 865 ± 197 mmol/l min, P < 0.0001) and postnatal HF diet (FE 1056 ± 120 mmol/l min, P < 0.0001), as well as in offspring of Cm dams (FE 544 ± 206 mmol/l min, P = 0.011). In planned comparison testing, Cm/HF offspring had significantly impaired glucose tolerance compared with C/HF offspring (FE 758 ± 265 mmol/l min, P = 0.006). By contrast, offspring of HF dams given metformin had significantly improved glucose tolerance (FE -858 ± 249 mmol/l min, P < 0.001). Fasted insulin level in females was markedly increased by maternal HF diet (FE 23.79 ± 6.72 mU/l, P < 0.001) and by postnatal HF diet (FE 18.63 ± 4.55 mU/l, P < 0.0001). Maternal metformin treatment in HF dams significantly reduced fasting insulin in offspring (FE -28.10 ± 9.84 mU/l, P = 0.008).

In males, glucose tolerance (Figure 16, Table 15) was only impaired by postnatal HF diet (FE 705 ± 135 mmol/l min, P < 0.0001). Despite no other main effects or interactions being significant, in planned comparison tests, Cm/C and Cm/HF groups both showed reduced glucose tolerance compared with C/C (FE 1105 ± 438 mmol/l min, P = 0.019) and C/HF (FE 1154 ± 443 mmol/l min, P = 0.016), respectively. Male fasted insulin concentration was significantly increased by the main effect of postnatal HF diet (FE 15.75 ± 6.90 mU/l, P = 0.028), although this effect seems to be primarily due to raised insulin levels in Cm/HF vs C/HF offspring (non-significant in planned comparison tests). There was a significant interaction between maternal and postnatal HF diets that dramatically increased offspring fasted insulin (FE 21.31 ± 9.53 mU/l, P = 0.031).

HOMA-IR, a proxy measure of insulin sensitivity, was markedly increased in females by maternal (FE 0.48 ± 0.13 logHOMA-IR, P < 0.001) and postnatal (FE 0.46 ± 0.09 logHOMA-IR, P < 0.0001) HF

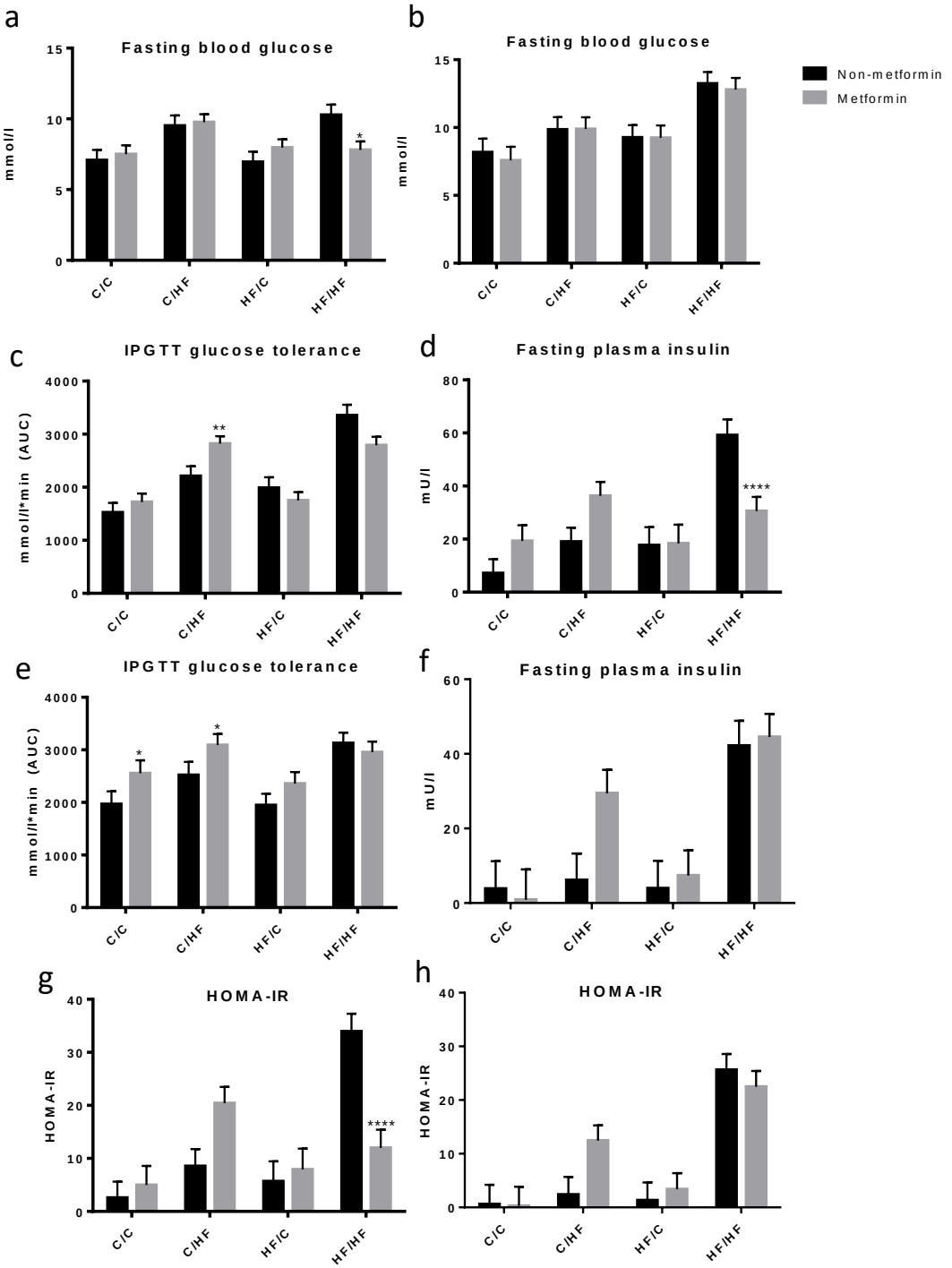

Figure 16 | **Measures of adult offspring glucose and insulin homeostasis**. **a.** FBG in female offspring. **b**. FBG in male offspring. **c.** Glucose tolerance in female offspring. **d.** Fasting plasma insulin in female offspring. **e**. Glucose tolerance in male offspring. **f**. Fasting plasma insulin in male offspring. **g.** HOMA-IR in female offspring. **h**. HOMA-IR in male offspring. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; ****, P < 0.0001 versus respective untreated diet group in planned comparisons tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat; HOMA-IR, homeostatic model assessment of insulin resistance.

diets. Maternal metformin treatment in HF dams decreased HOMA-IR (FE -0.58 ± 0.19 logHOMA-

IR, P = 0.005), although planned comparison testing demonstrated that this effect was entirely attributable to the decreased HOMA-IR in HFm/HF vs HF/HF offspring (FE -1.36 ± 0.68 logHOMA-IR, P < 0.0001).

In males, HOMA-IR was increased by postnatal HF diet (FE 0.41 ± 0.16 logHOMA-IR, P = 0.017) but not by maternal diet. However, combined postnatal and maternal HF diet significantly increased HOMA-IR (FE 0.76 ± 0.23 logHOMA-IR, P = 0.002), as did the combination of maternal metformin in C dams in tandem with postnatal HF diet (FE 0.84 ± 0.22 logHOMA-IR, P < 0.001).

Dependent variable	Significant effects	Fixed effect est.	P
Female fasting blood glucose	Postnatal HF diet	1.78 ± 048 mmol/l	< 0.0001
Male fasting blood glucose	Maternal HF diet	2.50 ± 0.68 mmol/l	< 0.001
	Postnatal HF diet	2.95 ± 0.64 mmol/l	< 0.0001
Female glucose tolerance	Maternal HF diet	865 ± 197 mmol/l/min	< 0.0001
	Postnatal HF diet	1,065 ± 120 mmol/l/min	< 0.0001
	Metformin * Postnatal HF diet	544 ± 206 mmol/l/min	= 0.011
	Metformin * Maternal HF diet	-858 ± 249 mmol/l/min	< 0.001
Female fasting insulin	Maternal HF diet	23.79 ± 6.72 mU/l	< 0.001
	Postnatal HF diet	18.63 ± 4.55 mU/l	< 0.0001
	Metformin * Maternal HF diet	-28.10 ± 9.84 mU/l	= 0.008
Male glucose tolerance	Postnatal HF diet	705 ± 135 mmol/l/min	< 0.0001
Male fasting insulin	Postnatal HF diet	15.75 ± 6.90 mU/l	= 0.028
	Maternal HF diet * Postnatal HF diet	21.31 ± 9.53 mU/l	= 0.031
Female HOMA-IR	Maternal HF diet	0.46 ± 0.13 logHOMA-IR	< 0.001
	Postnatal HF diet	0.46 ± 0.09 logHOMA-IR	< 0.0001
	Metformin * Maternal HF diet	0.58 ± 0.19 logHOMA-IR	= 0.005
Male HOMA-IR	Postnatal HF diet	0.41 ± 0.16 logHOMA-IR	= 0.017
	Maternal HF diet * Postnatal HF diet	0.75 ± 0.23 logHOMA-IR	= 0.002
	Metformin * Postnatal HF diet	0.84 ± 0.22 logHOMA-IR	< 0.0001

Table 15 | Statistical results for Figure 16. HF, high-fat; HOMA-IR, homeostatic model assessment of insulin resistance.

5.3.5 Plasma levels of inflammation-related molecules in female offspring

Because the metabolic syndrome and obesity are associated with a chronic, low-level pro-inflammatory response that increases risk of comorbidities, levels of pro-inflammatory proteins were measured in the plasma of female offspring (Figure 17, Table 16). On the basis of the lack of beneficial effect on glucose homeostasis of metformin in male offspring of HF-dams, and a similar response in male offspring of metformin-treated C-dams to that in female offspring, levels were only assessed in female offspring. Levels of IFNγ were substantially increased by maternal HF diet (FE 0.8 ± 0.35 pg/ml, P = 0.035) and by postnatal HF diet (FE 0.75 ± 0.33 pg/ml, P = 0.032). Notably, HF/HF offspring had reduced levels compared with HF/C and C/HF animals, suggesting a protective effect with the combination of maternal and postnatal HF diets (maternal HF diet x postnatal HF diet interaction FE -2.03 ± 0.46 pg/ml, P < 0.0001). The reduction between C/HF and Cm/HF groups was significant in planned comparison testing (FE -1.35 ± 0.41 pg/ml, P = 0.003).

CRP, a marker of inflammation elevated in obesity, was increased by postnatal HF diet (FE 2.37 ± 0.73 mg/l, P = 0.003), although this effect was largely attributable to increased levels in Cm/HF mice. Exposure to both maternal and postnatal HF diets significantly increased CRP levels (maternal HF diet x postnatal HF diet interaction FE 2.44 ± 1.01 mg/l, P = 0.024). In planned comparison testing, the increased CRP level in Cm/HF vs C/HF animals was significant (FE 2.63 ± 1.18 mg/l, P = 0.038).

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Plasma IFNγ (females)	Maternal HF diet	0.80 ± 0.35 pg/ml	= 0.035
	Postnatal HF diet	0.75 ± 0.33 pg/ml	= 0.032
	Maternal HF diet * Postnatal HF diet	-2.03 ± 0.46 pg/ml	< 0.0001
Plasma CRP (females)	Postnatal HF diet	2.37 ± 0.73 mg/l	= 0.003
	Maternal HF diet * Postnatal HF diet	2.44 ± 1.01 mg/l	= 0.024
Plasma IL-6 (females)	Postnatal HF diet	8.9 ± 4.2 pg/ml	= 0.041
Plasma IL-10 (females)	Metformin	-3.7 ± 1.7 pg/ml	= 0.033
Plasma TNF (females)	NONE	NA	NA

Table 16 | Statistical results for Figure 17. HF, high-fat. NA, not applicable.

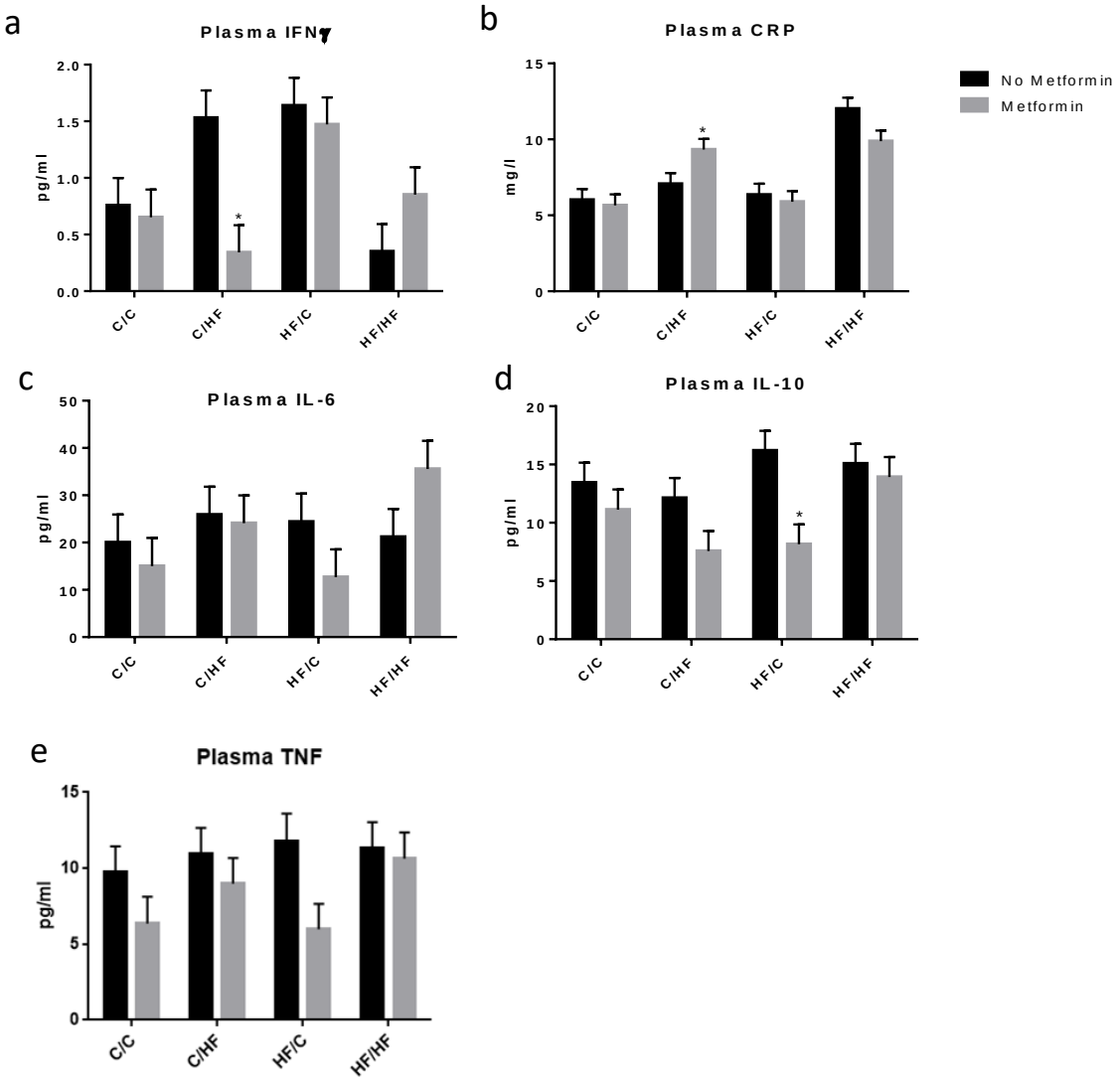

Figure 17 | **Levels of plasma markers of inflammation in adult female offspring.** Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text *, P < 0.05 vs respective nontreated diet group in planned comparison tests. C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). C, chow; HF, high-fat.

Although numerous interleukin family protein levels were assessed (IL-2, IL-1β, IL-5, IL-6, IL-10 and IL-12), only two (IL-6 and IL-10) were significantly affected by any factors. IL-6, which increases insulin secretion but is elevated in those with obesity, was increased by postnatal HF diet (FE 8.9 ± 4.2 pg/ml, P = 0.041) but was otherwise unaltered. IL-10, which acts a potent inhibitor of inflammation (including suppressing the synthesis of pro-inflammatory TNF and IL-6), was unaltered except by metformin treatment, which significantly reduced IL-10 levels (FE -3.7 ± 1.7 pg/ml, P = 0.033). This effect was seen across all groups, but was most pronounced in HFm/C vs

HF/C offspring, the only significant pairwise planned comparison (FE -8.1 ± 2.6 pg/ml, P = 0.003). TNF levels were unaltered across all groups.

5.4 Discussion

A substantial body of prior work has demonstrated the diverse and often powerful effects that priming via maternal obesity during conception and early-life can have on offspring. Data presented in this chapter show clear effects of maternal HF diet and maternal obesity on adult offspring, in a manner dependent on both offspring sex and postnatal diet. Moreover, metformin treatment can alter the subsequent adult phenotype. In contrast to other models, offspring in this study were assessed at 30 weeks of age, rather than at around 15 weeks. In other studies using the same maternal obesity model reported here, metabolic dysfunction is exacerbated by age[269]. As the intention of this model is to explore the potential effects of early-life metformin treatment in humans, offspring were studied at an age at which metabolic changes resulting from maternal obesity are more evident[269]. In addition to a later age of assessment, all offspring groups were included in statistical analyses, allowing more direct comparison of the differential priming effect of metformin in lean and obese dams.

In humans, exposure to maternal obesity *in utero* is associated with large-for-gestational-age at birth and increased adiposity in childhood. These effects have been observed also in rodent models, with neonatal animals being heavier and possessing more adipose tissue.

Degree of adiposity and body weight gain was exacerbated in adult female offspring of obese dams, compared with female offspring of normal weight dams. Maternal diet independently and markedly increased female offspring body fat proportion, with HF/C females showing around a twofold greater WAT mass than C/C females. These data are in good agreement with published work in similar rodent models[349]. These effects on offspring adiposity were different in male offspring, in which the independent effect of maternal HF diet on offspring body weight was not observed; however, maternal diet did exacerbate the effect of postnatal HF diet. The multiplicative interaction between maternal and postnatal HF diets, inducing increased offspring body weight, has also been observed in other rodent studies[362]. The dramatic effect of combined maternal and postnatal HF diets was also observed in WAT proportion and in µCT-assessed body fat percentage. The association of *in utero* obesity exposure and expanded visceral adipose tissue has been documented in previous human epidemiological studies[363] and maternal obesity animal models, such as the substantially increased perirenal fat pad mass reported by Bayol *et al*. in junk-food-fed female offspring of obese dams, compared with female offspring only exposed to postnatal junk food diet[364]. As increased visceral fat is linked to worsened cardiometabolic outcomes,

driven in part by insulin resistance and systemic inflammation[15], proportion of visceral adipose tissue was assessed in male and female offspring. Maternal HF diet independently increased visceral adipose tissue mass and visceral adipose tissue proportion relative to subcutaneous adipose tissue in female offspring, in agreement with the results of Bayol *et al.*, yet there were no statistically significant effects in male offspring. iBAT, relative expansion of which has been associated with improved outcomes during obesogenic feeding in animal models, was increased in male and female offspring only by combined maternal and postnatal HF diets. Adiposity responses in male and female offspring as a result of maternal HF diet feeding are therefore broadly similar; however, substantial sex-specific differences were seen in the response to metformin treatment.

In female offspring of HF dams, metformin treatment was generally protective, irrespective of postnatal HF diet. Metformin in these groups reduced offspring body weight and decreased the degree of adiposity and amount of visceral adipose, with planned comparison tests showing that the HFm/C group benefited most. By contrast, male offspring adiposity was not reduced by maternal metformin treatment. These results hint at a sex-specific persistent response to maternal metformin treatment *in utero*, independent of sex-specific responses to obese pregnancy. Whether these effects are caused by the direct acute action of metformin on male offspring *in utero* being different from the action in female offspring, or whether female fetuses are more responsive to improved maternal metabolic parameters during pregnancy, is not clear. Sexual dimorphism in the priming of offspring metabolism by maternal obesity is well-appreciated[258]. Salomaki *et al.* observed clear sex differences in offspring of metformin-treated HF-fed dams, whereby female offspring of metformin-treated HF-diet-fed dams had reduced body fat % than female offspring of untreated HF-diet-fed dams – there was no difference seen between male offspring of treated or untreated HF-diet-fed dams[255]. Although the mechanisms underlying sex-specific differences are under-researched, the response of the placenta to maternal obesity or high-fat diet has been demonstrated to differ based on offspring sex in mice[365,366] and humans[367]. In particular, male human fetuses and associated placentas have been suggested to respond less to *in utero* insults, such as nutrient restriction or preeclampsia, rendering them vulnerable if the insult continues[367]. Thus, the sex differences observed might reflect the greater response of female fetuses and placentas to improved maternal metabolic homeostasis with metformin, rather than an interaction between metformin and sex hormones during development. In a trial of women given metformin or placebo for PCOS during pregnancy, metformin had no effect on offspring androgen and oestrogen levels at birth[368].

Although metformin exerted neutral or beneficial effects on the degree of offspring adiposity in those born to treated HF-fed dams (HFm/C and HFm/HF males and females), body weight and body fat was increased in offspring born to Cm dams. These effects were particularly exacerbated by postnatal HF diet insult in Cm/HF animals; both male and female Cm/HF offspring showed increased body fat, with male Cm/HF mice having the highest body fat of all groups. Notably, this

data is similar to that reported by Salomaki *et al.*, who reported that maternal metformin in lean pregnancy increased body weight gain in both male and female offspring when they were fed a HF diet[253]. There was also a tendency towards increased body fat in both sexes, and significantly increased mesenteric fat pad mass. Although their model is not identical to that reported here (aside from diet composition, the maternal metformin dose employed was 300mg/kg, rather than 250mg/kg; offspring received postnatal HF diet from 10 weeks of age, rather than from weaning; and offspring were sampled at 20 weeks of age, rather than 30 weeks), the results reported here are markedly similar. The Finnish group noted the similarity of the phenotype of metformin-exposed offspring of C-fed dams to an intrauterine growth restriction phenotype[253]. Intrauterine growth restriction via maternal undernutrition is a potent risk factor for offspring metabolic disease; indeed, the association between stroke and coronary heart disease and low birth weight, resulting from nutrient restriction *in utero*, was one of the first observations of developmental priming of chronic disease[118].

Animal models have demonstrated increased adiposity in offspring as a result of maternal nutrient restriction throughout pregnancy. Howie *et al*., for instance, showed increased adiposity in adult male and female offspring of female rats subjected to 50% nutrient restriction during pregnancy, despite weaning all offspring on to a C diet[369]. Other studies have shown that exposure to maternal nutrient deprivation increases hyperphagia via alteration of central appetite networks, including increased expression of hypothalamic neuropeptide Y, an orexigenic neuropeptide, and increased circulating leptin levels[370]. Metformin has been found to exert calorie-restriction-like effects of gene expression in *Caenorhabditis elegans*[371] and it's chronic use at low doses in lean mice markedly reduced fasting plasma insulin levels, HbA1c and low-density lipoprotein cholesterol levels, albeit not to the levels achieved with chronic calorie restriction[372]. Interestingly, despite these metabolic improvements, fasting blood glucose was unaltered by metformin. Together, these data are persuasive that the effects of metformin in lean dams are the result of a caloric-restriction-like state in the mother or in the fetus, via effects on the dam and placenta.

One limitation of the data presented here is that the causative source of weight gain could not be accurately assessed due to data recording issues with the automated scales used to measure food consumption. Moreover, energy expenditure could only be measured in a few offspring, making assessment of any change in either total energy expenditure or individual energy expenditure components difficult. Appetite has been found to be increased in offspring of obese dams in many models[152], and is probably the primary mechanism driving increased adiposity in offspring of HF dams in this model. Energy expenditure might also be decreased in the offspring of HF dams[182], yet such measurements require accurate and appropriate adjustment for body composition, as

energy expenditure per g of bodyweight is confounded by the increased proportion of body fat in these offspring[340].

Although no changes in blood pressure were observed in male offspring as a result of either diets or metformin treatment, female offspring showed increased SBP as a result of exposure postnatal HF diet. There was a significant interaction between maternal and postnatal HF diets, indicating reduced SBP as a result of a combined exposure relative to the independent effect of postnatal or maternal HF diets. This interaction represents the lack of an additive effect of postnatal and maternal HF diets on SBP, and does not necessarily imply a predictive adaptive response (whereby suboptimal early life nutrition results in offspring benefit if the suboptimal nutrition continues into postnatal life, compared with offspring receiving improved nutrition in adulthood)[356]. As such, these data do not strongly support an effect of maternal or postnatal HF diets, or metformin treatment, on SBP or DBP in this model. Despite the lack of effect on blood pressure, raw heart mass was significantly increased in male offspring by both maternal and postnatal HF diets, although the increase in HF/C vs C/C offspring was marginal. Fernandez-Twinn *et al.* similarly documented heavier hearts in male offspring of HF-fed dams, before offspring were obese, indicating a priming effect independent of adiposity[179]. Interestingly, in this study, metformin decreased heart weight in HFm/HF compared with HF/HF males, despite no body weight or adiposity differences between these groups. This observation supports a metformin-specific priming effect on the prevention of cardiac hypertrophy, although more studies are required to investigate this aspect.

Effective glucose homeostasis is critical for metabolic health. The data presented here demonstrate sex-specific effects of maternal obesity and metformin treatment on offspring glucose homeostasis. Although FBG concentration in females was unaltered by maternal diet, fasting insulin levels were substantially increased, indicating a greater required level of insulin to maintain FBG at physiological levels. This finding is emphasised by the markedly increased HOMA-IR index, validated as an indirect measure of insulin resistance, as a result of maternal HF diet in female offspring. These data are similar to those reported in human offspring of mothers who are obese and offspring in animal models of obesity[70,172]. The pattern of effects in males was different from that seen in females; FBG was significantly increased by maternal and postnatal HF diets, whereas there was no independent effect of maternal HF diet on glucose tolerance. Although there was no effect of maternal HF diet on fasting insulin, there was a strong effect of combined maternal and postnatal HF diets, as evidenced by increased levels in HF/HF offspring. Other animal models have demonstrated sex-specific obesity-mediated programming effects on offspring glucose tolerance, with groups showing decreased tolerance[172,349] in male offspring or decreased tolerance in female offspring[355]. Fasting plasma insulin was increased in males by

postnatal HF diet, yet this effect seems to be observed only in offspring groups exposed to both maternal and postnatal HF diets, and the Cm/HF offspring. HOMA-IR index in males also followed this pattern, with a significant independent effect of postnatal HF diet and a combined interaction effect in HF/HF groups.

The priming effect of metformin on glucose homeostasis in offspring of HF dams was disparate between the sexes, with HF/HF female offspring being afforded greatest protection from impaired FBG, glucose intolerance and insulin resistance. Interestingly, in contrast with the beneficial effects on female adiposity seen in HFm/C but not HFm/HF offspring, glucose homeostasis was not improved in HFm/C vs HF/C offspring. HOMA-IR was dramatically reduced in HFm/HF vs HF/HF females, although it did remain elevated relative to the C/C group. Strikingly, the improved markers of glucose homeostasis in HFm/HF offspring were independent of any effect on offspring adiposity, suggesting an effect on other tissues relevant to whole-body glucose control. As the liver is a primary source of FBG, via gluconeogenesis, these data suggest that hepatic insulin sensitivity might be improved in these animals. Given the importance of skeletal muscle, the liver and adipose in taking up glucose to maintain glucose homeostasis, metformin could be priming improved responses to the glucose bolus (that is, glucose uptake) in any of these tissues. In line with this notion, Tong *et al.* demonstrated that metformin treatment in obese dams protected offspring from decreased mitochondrial copy number, improved AKT phosphorylation (a downstream marker of insulin signalling) during IPGTT and increased AMPK phosphorylation in skeletal muscle[145]. Delineating the exact mechanisms by which glucose tolerance is improved in the model reported here will require further investigation of peripheral tissues.

In males, metformin had no effect in offspring of HF dams. Salomaki *et al.* showed impaired glucose tolerance in offspring (both male and female) of HF dams challenged with a HF diet postnatally; however, glucose tolerance was not impaired by the HF diet challenge in offspring of obese dams treated with metformin, suggesting that metformin protects against the increased glucose intolerance induced by postnatal HF diet[255]. This effect was demonstrated in this chapter in female offspring, but not in males. As discussed above, the decreased male plasticity in response to changing *in utero* conditions could underlie this disparity.

However, the detrimental effect of metformin in leans dams, especially in those given a postnatal HF diet, was frank and seen in both male and female offspring. Glucose tolerance was impaired in Cm/HF versus C/HF offspring in both sexes, and there was also a trend towards increased insulin resistance in these groups. Salomaki *et al*. did not observe any changes to glucose tolerance in offspring of lean, metformin-treated dams, either during the C-diet phase or the HF-diet, although their model assessed offspring at 10 to 15 weeks of age[253]. Thus, the data reported here are

better representative of the long-term effects of metformin priming and chronic, life-long exposure to a postnatal HF diet. As such, male Cm/C offspring were found to have significantly impaired glucose tolerance relative to C/C offspring; the model reported by Salomaki showed no effect of metformin in males, highlighting the importance of age effects in the manifestation of metabolic disease.

Finally, the effects of postnatal diet, maternal diet and metformin were observed on offspring levels of circulating inflammatory markers. Notably, levels of CRP, which are elevated in human obesity and in offspring of obese mothers[373] and reflect a low-grade inflammatory state, were increased by the independent effect of maternal HF diet, and exacerbated substantially by postnatal HF diet as a secondary insult in HF/HF offspring. Metformin increased CRP levels in Cm/HF offspring, in line with the broad metabolic defects in this group discussed above. Given the strong association between CRP levels and degree of adiposity[374], it is probable that this increase is associated with increased fat mass in this group. Although levels of IFNγ were elevated by maternal and postnatal HF diets, they were markedly reduced in Cm/HF animals compared with C/HF animals, and levels in HF/HF and HFm/HF groups were similar to controls. Why IFNγ is reduced in the groups showing the highest degree of metabolic disease is unclear. Serum levels of the protein are increased in overweight and obese humans and chimpanzees[375]. However, extreme obesity in humans has been shown to impair lipopolysaccharide-stimulated IFNγ production by peripheral blood mononuclear cells[376]; as such, the results here might be due to the relatively extreme phenotype in HF/HF, HFm/HF and Cm/HF groups. Levels of IL-10 are associated with increased insulin sensitivity, and are decreased in individuals with obesity and diabetes. The reduction as a result of metformin treatment did not differ based on maternal or postnatal diets, suggesting a priming effect independent of adiposity or metabolic health in this model.

In summary, the data reported here demonstrate the long-term, chronic effects of maternal metformin exposure during gestation and lactation on offspring. In contrast to the model employed by Salomaki, the only other prior report of the developmental priming effects of metformin in adult offspring, offspring were aged to 30 weeks to better expose deficits in metabolic homeostasis that are relevant to human exposures. Given the lack of human data on the long-term effects of metformin in pregnancy, experiments to explore these effects are critical. While studies of metformin in mothers with obesity without overt gestational diabetes have failed to show any benefit in terms of birth weight or total offspring adiposity[243,377], the results presented here suggest that beneficial effects might manifest in the long-term. However, the exacerbation of metabolic dysfunction in offspring of lean mothers urges caution against indiscriminate treatment of obese mothers with metformin in clinical practice. HF dams reported here were moderately glucose intolerant (Chapter 4); whether metformin in obese dams without

glucose intolerance might result in a maternal undernutrition phenotype and subsequent priming of metabolic disease, as seen in Cm/HF offspring, is unknown. Whereas elevated blood glucose might provide a 'buffer' against reduced fetal fuel delivery mediated by metformin in mothers with overt GDM, metformin treatment in obese mothers with normal FBG and blood glucose tolerance could restrict nutrient transfer to the fetus.

Chapter 6: Metformin alters priming of male and female liver physiology by maternal obesity

6.1 Introduction

Although maternal obesity is associated with organism-wide developmental priming effects on many organs, the mammalian liver seems to be particularly vulnerable to early life insults[378]. These findings are worrisome, and the mechanisms responsible might underlie, in part, the increasing prevalence of NAFLD in children[379] and adults[380].

NAFLD is a spectrum of liver disease covering the full range of presentations between simple steatosis, NASH, liver cirrhosis and HCC. Examination of liver biopsy samples remains the gold standard diagnosis. Simple steatosis (NAFL) is defined by the presence of steatosis, macrovesicular and/or microvesicular in nature, occupying >5% of the histologically assessed biopsy area[26]. The presence of an inflammatory lymphocyte infiltrate, along with ballooning degeneration of hepatocytes, are critical features indicative of NASH[26]. Fibrosis in NASH, visualised by Masson's trichrome stain or Sirius red stain, consists of the deposition of extracellular matrix, produced by activated hepatic stellate cells. The presence and severity of fibrosis is associated with poorer clinical outcomes[381]. The severity of NAFLD can be assessed using systems that score features of the disease present in biopsy samples; the NAFLD activity score (NAS) is the most widely used and best validated histological scoring system[26] (Figure 18).

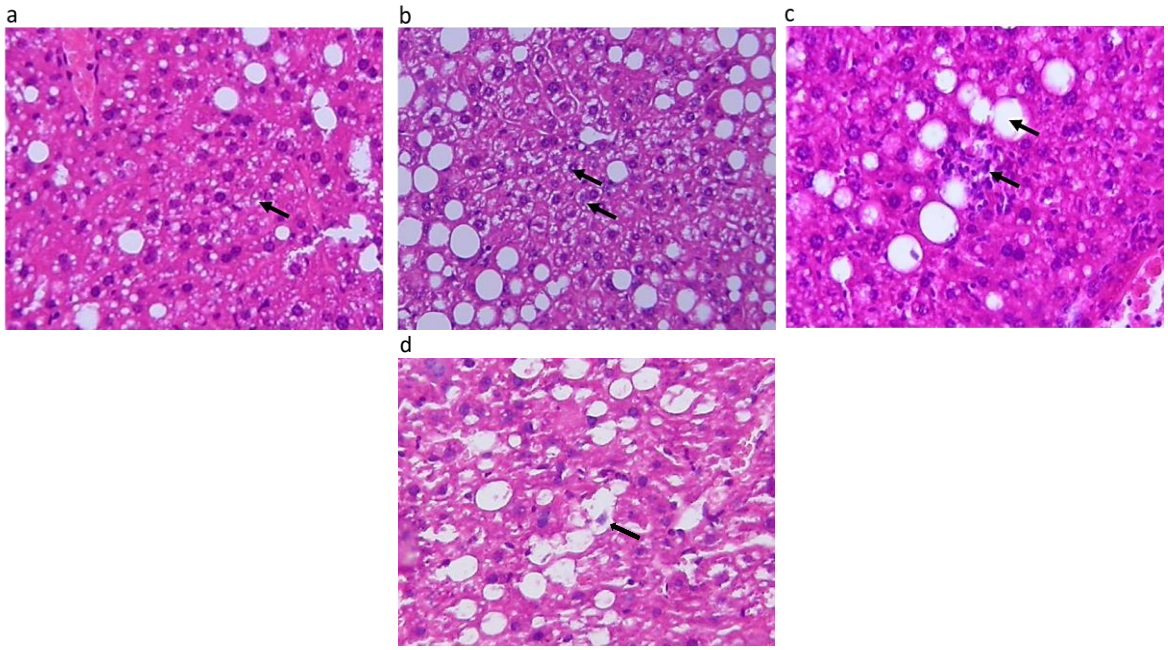

Figure 18 | **Illustration of NAS components**. **a**. HF/C female mouse showing mild macrovesicular steatosis and mild microvesicular steatosis (arrow). **b**. C/HF female mouse showing moderate/severe macrovesicular steatosis and microvesicular steatosis (arrows). **c**. HF/HF female mouse showing an infiltrating leukocytes (lower arrow) and macrovesicular steatosis (upper arrow). **d**. HF/HF female mouse showing a ballooned hepatocyte (arrow). Magnification 200x, stained with H+E. C, chow; HF, high-fat; NAS, NAFLD activity score.

Maternal obesity is associated with the development and severity of NAFLD in offspring. Bruce *et al.*[70] found that offspring exposed to maternal obesity *in utero* and a postnatal HF diet had NASH at 15 weeks of age, characterized by marked steatosis and lobular inflammation. By 30 weeks of age, steatosis was more extensive, and there was also prominent hepatocyte ballooning. By contrast, NASH was much less severe in offspring only exposed to postnatal HF diet at both time points. Notably, offspring only exposed to maternal obesity had developed NAFLD by 30 weeks. similar findings in mouse models were reported by Mouralidarane *et al.* in animals at 12 months of age[286], and by Pruis *et al*. in animals at 29 weeks of age [382]. Numerous studies have demonstrated that maternal obesity primes increased steatosis or hepatic TAG content in offspring[148,286,287,382]. Mouralidarane *et al.* also found increased fibrosis in offspring of obese dams fed a postnatal HF diet, although the independent contribution of maternal obesity to fibrosis development was not statistically significant.

NAFL has classically been regarded as benign with little risk of progression, yet data in the past few years from serial biopsy studies have suggested progression to NASH in patients with baseline NAFL is a relatively common occurrence. McPherson *et al.* found progression to NASH from NAFL in 44% of patients[25]. Pais *et al.* showed similar course of progression, with 64% of those with baseline NAFL progressing to NASH over a mean of 3.7 years[383]. These findings render any mechanism that increases the degree of steatosis concerning. Combined with evidence supporting indirect and direct means by which maternal obesity *in utero* promotes NAFL in offspring, these observations demonstrate the importance of mitigating the developmental priming mediated by overnutrition and obesity during pregnancy.

Studies investigating the impact of interventions during obese pregnancy on offspring NAFLD risk are lacking. Heerwagen *et al.* used a transgenic mouse model (*Fat-1* mice), which are capable of converting n-6 PUFAs to n-3 PUFAs, to investigate how increasing maternal n-3 PUFA levels alter developmental priming of offspring associated with obesity. Wild-type offspring of obese Fat-1 mice showed lower levels of hepatic TAG than wild-type offspring of wild-type obese mice, despite Fat-1 obese dams being the same weight as wild-type dams. Antioxidants have also been used in mice models of obese pregnancy, with offspring of obese dams treated with antioxidants showing reduced body fat and normalized glucose tolerance[194]. Improvement in these parameters is associated with reduced risk of NAFLD, yet this was not assessed in this study. Although they did not gauge severity of NAFLD in offspring, Salomaki *et al.* showed that metformin treatment in obese dams improved glucose tolerance and reduced adiposity in offspring, especially in females[255]. In another study, fetal livers exposed to maternal obesity *in utero* displayed increased inflammation, greater SFA and MUFA levels and reduced levels of n-3 PUFAs. Maternal metformin treatment in this model reduced hepatic inflammation and levels of TAG-fraction MUFA, and

restored TAG-fraction n-3 PUFA levels, despite not influencing total hepatic TAG[256]. Maternal exercise, a potentially powerful modifier of maternal-obesity-mediated priming yet limited by adherence and uptake, significantly increased offspring insulin sensitivity in multiple models[384,385].

Together, these data imply the ability of interventions in animal models to modulate offspring insulin sensitivity and adiposity, although hepatic outcomes in maternal obesity intervention models are under-researched. Of potential pharmaceutical interventions, metformin has received the most attention in human clinical trials, owing to demonstrable safety in short-term maternal and neonatal outcomes. Although metformin did not reduce incidence of increased neonate body weight in the EMPOWaR[243]or MOP[244] studies, longer-term metabolic or hepatic outcomes have not been assessed in humans and therefore require investigation. Use of the model employed by Bruce *et al.* enables the assessment of the effects of metformin in a mouse model in which offspring of obese dams develop more severe NAFLD than those not exposed to maternal obesity[70].

6.2 Methods

6.2.1 Animal model

Offspring were generated and tissue collected according to the protocol described in detail in Chapter 3. Briefly, female C57BL/6 mice received either a HF or a C diet for 6 weeks prior to mating. Immediately after successful mating, half of dams from each group received metformin (250mg/kg/day) dissolved in drinking water, generating four maternal groups: C, Cm, HF and HFm. Metformin treatment and respective maternal diets were continued throughout pregnancy and lactation. Offspring were weaned at 4 weeks onto C or HF diet, producing eight offspring groups denoted by maternal diet, metformin treatment and postnatal diet: C/C, Cm/C, C/HF, Cm/HF, HF/C, HFm/C, HF/HF and HFm/HF. Offspring of both sex were killed at 30 weeks of age, and livers were weighed and the left lobes bisected. One half was snap frozen in liquid N_2 and used for gene expression analysis. A sample of left liver lobe was also fixed in 10% formalin for 24 hours for histological assessment of NAFLD.

6.2.2 Liver histology

Fixed adult offspring liver from was processed for liver histology as described in Chapter 3. Mounted 4μm-thick liver sections were stained with haemotoxylin and eosin (for assessment of NAS or steatosis) or Sirius red (for assessment of liver fibrosis).

6.2.2.1 NAS

Blinded liver sections from offspring were scored according the NAS developed and validated by Kleiner *et al.*[285], according to the method described in detail in Chapter 3.

6.2.2.2 Quantification of steatosis

Degree of hepatic steatosis was quantified in liver sections from female adult offspring in a blinded fashion, according to the method described in detail in Chapter 3. Briefly, a point counting system was employed on 10 independent liver histology images from each offspring, and percentage of hepatic area occupied by steatosis calculated.

6.2.2.3 Qualitative assessment of liver fibrosis

Sirius-red-stained liver sections from adult female offspring were assessed for degree of liver fibrosis in a blinded fashion as described in detail in Chapter 3. Briefly, liver sections were scored by fibrosis scoring by the scoring methodology described by Kleiner *et al*[285].

6.2.3 Hepatic gene expression analysis

Expression of genes related to inflammation in female adult offspring was assessed according to the detailed methods described in Chapter 3. Briefly, mRNA was extracted from snap-frozen liver via homogenization with Tri reagent. cDNA synthesis was performed using M-MLV reverse transcriptase from Promega, UK, according to manufacturer's instructions.

As described in Chapter 3, real-time qPCR was performed using premixed qPCR MasterMix and pre-optimised TAQMAN probes specific to genes of interest, both by Eurogentec, UK, on an ABI 7500 real-time PCR system with reaction conditions as specified by the manufacturer. Gene expression was normalized against *Ywhaz* levels (previously validated as a stable housekeeping gene in this model[282]) and expression of the target gene in C/C animals by the ΔΔCt method.

6.2.4 Statistical analysis

Mixed models were constructed in SPSS v24 to assess the individual effects of maternal HF diet, maternal metformin treatment and postnatal HF diet, as well as interactions between these factors, on measured dependent variables in offspring, as described in Chapter 3. Ct values derived from PCR data were logged prior to statistical interrogation. All data is presented as graphs and/or tables of predicted mean ± SEM from maximal models (containing all interactions to maximise model fit), produced using GraphPad Prism v6.0.1. PCR data is graphed from non-logged predicted means ± SEM for clarity, although statistical analysis was performed on logged data. Normality of other variables was assessed as described in Chapter 3, and non-normal variables were log transformed for statistical analysis.

Tables accompany each figure to report and summarize significant fixed effects for each respective dependent variable.

Planned comparison tests were also conducted between respective treated and non-treated offspring groups, as detailed in Chapter 3. Data from these tests are reported in the main text and shown in graphs using asterisks.

6.3 Results

6.3.1 Gross liver physiology

Raw liver weight in females (Figure 19, Table 17) was significantly increased by maternal HF diet (FE 0.42 ± 0.01g, P < 0.0001) and by postnatal HF diet (FE 0.19 ± 0.06g, P = 0.003). Furthermore, combined exposure to maternal and postnatal HF diet increased liver weight (FE 0.39 ± 0.17g, P = 0.028). Maternal metformin treatment in HF dams reduced offspring liver size (FE 0.53 ± 0.12g, P < 0.0001), yet this effect was due to the markedly smaller HFm/HF livers vs HF/HF livers (FE -0.53 ± 0.12g, P < 0.0001 in planned comparison tests). Cm/HF livers were larger than C/HF livers (FE 0.30 ± 0.14, P = 0.041). When expressed as a % of body weight, only postnatal HF diet decreased liver size (FE -0.84 ± 0.26 % points, P = 0.002), although in planned comparison tests the difference between HFm/HF and HF/HF liver sizes was highly significant (FE –0.89 ± 0.26 % points, P < 0.001).

Comparatively, in males, raw liver weights were increased only when maternal and postnatal HF diets were combined (FE 0.80 ± 0.17g, P < 0.0001), with no effect of metformin treatment in

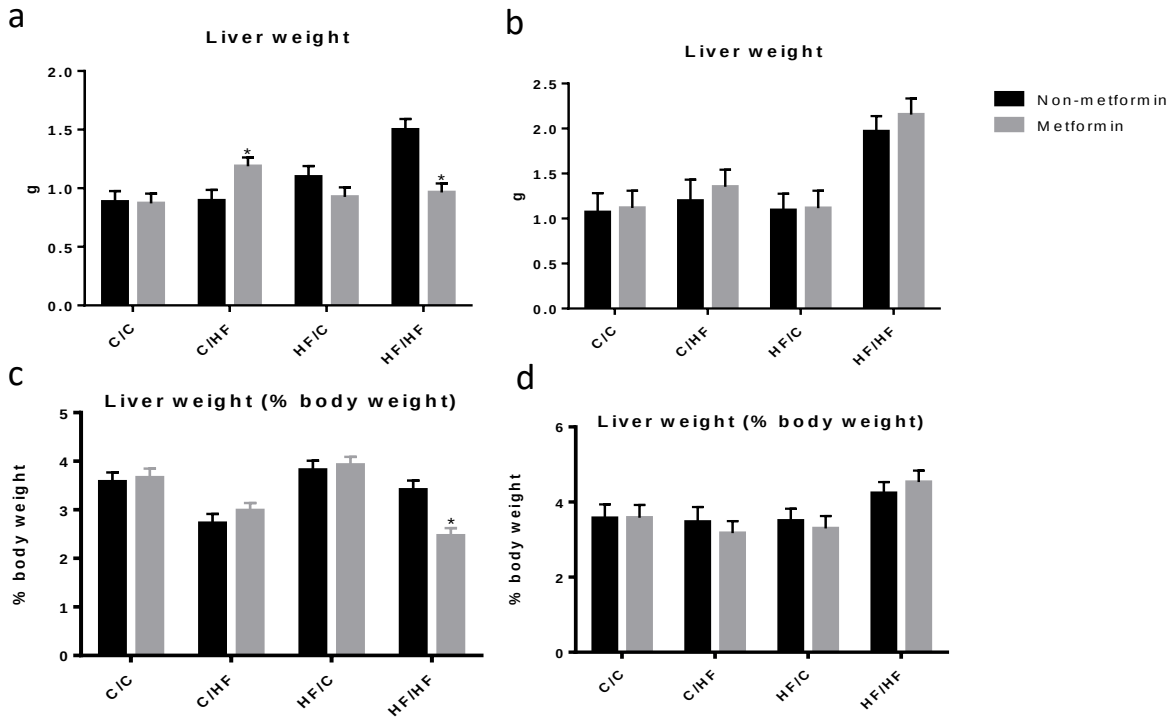

Figure 19 | **Raw and adjusted liver weights in adult offspring. a**. Adult female liver weight. **b**. Adult male liver weight. **c**. Adjusted adult female liver weight**. d**. Adjusted adult male liver weight. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective untreated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat.

HFm/HF or Cm/HF offspring. The effect of combined maternal and postnatal HF diet remained significant when liver weights were adjusted for body weight (FE 1.37 ± 0.34 % points, P < 0.0001).

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Female liver mass	Maternal HF diet Postnatal HF diet Maternal HF diet * Postnatal HF diet Metformin * Maternal HF diet	0.42 ± 0.01 g 019 ± 0.06 g 0.39 ± 0.17 g 0.53 ± 0.12 g	< 0.0001 = 0.003 = 0.028 < 0.0001
Liver mass % body weight (females)	Postnatal HF diet	-0.84 ± 0.26 % points	= 0.002
Male liver mass	Maternal HF diet * Postnatal HF diet	0.80 ± 0.17 g	< 0.0001
Liver mass % body weight (males)	Maternal HF diet * Postnatal HF diet	1.37 ± 0.34 % points	< 0.0001

Table 17 | Statistical results from Figure 19. HF, high-fat

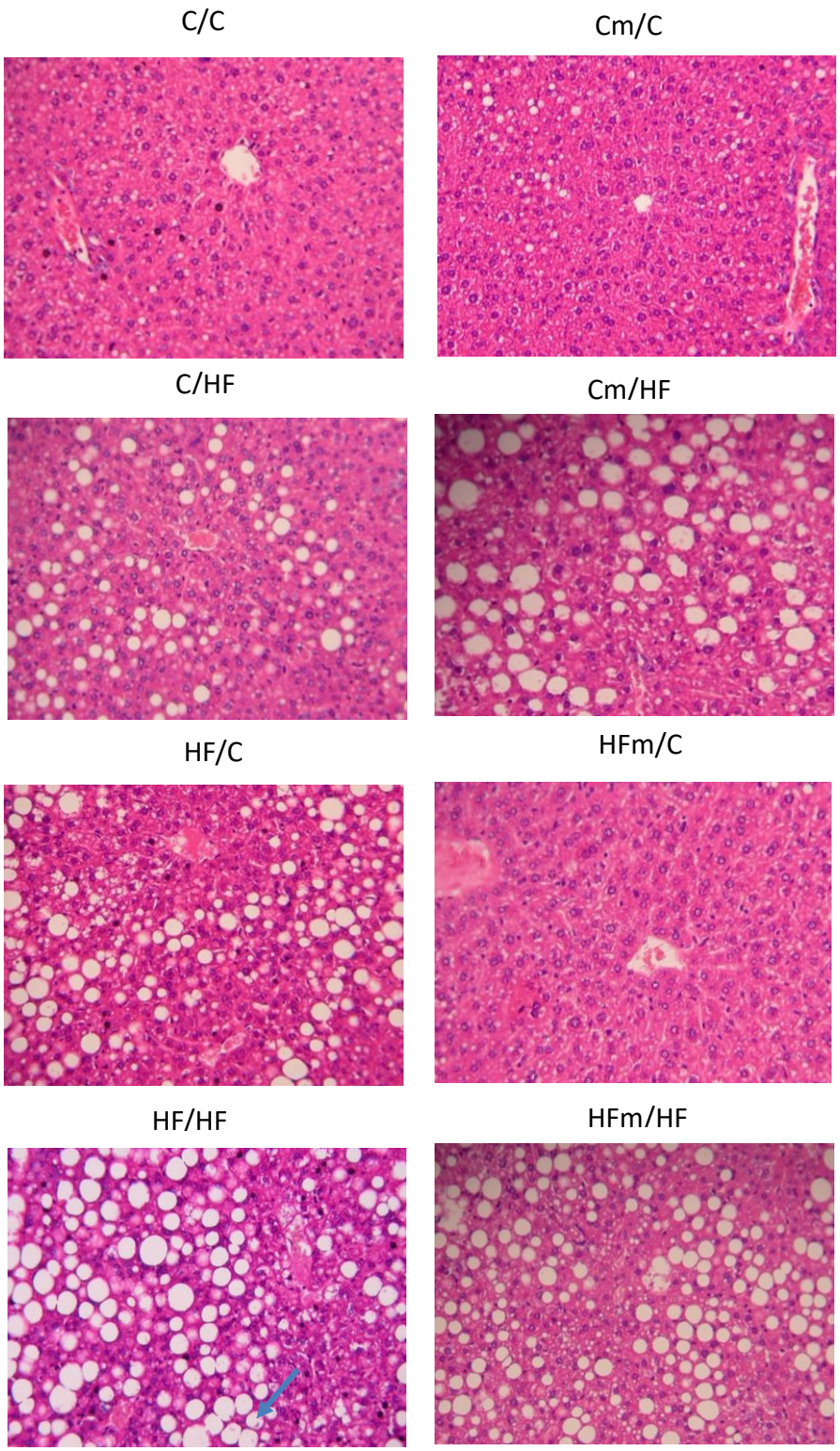

Figure 20 | **Haemotoxylin and eosin stained liver sections from adult female offspring.** Images are representative of typical liver pathology within each group. Only C/C and HFm/C sections show normal liver pathology. Extensive steatosis is visible in C/HF, Cm/HF, HF/C, HF/HF and HFm/HF females. Some hepatocyte ballooning is visible in the HF/HF group (arrow). Magnification 200x. Total number of liver assessed per group: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). C, chow; HF, high-fat

6.3.2 Histological assessment of NAFLD in adult female offspring

NAFLD severity was assessed blindly by NAFLD activity score (NAS) on H+E stained liver sections (Figure 20), giving a total mean score (from a maximum of 8 points) for each group and mean scores for each subsection (inflammatory foci (0–3), hepatocyte ballooning (0–2) and steatosis (0–3)). In female offspring, total NAS (Figure 22, Table 18) was independently increased by both maternal (FE 1.9 ± 0.7 points, P = 0.007) and postnatal (FE 2.4 ± 0.5 points, P < 0.0001) HF diets, with NAS exacerbated in HF/HF animals exposed to both diets but only in an additive, and not multiplicative, manner (interaction between maternal and postnatal HF diets, P = 0.87). Moreover, NAS was significantly reduced in offspring of HF dams given metformin (FE -2.2 ± 0.7 points, P = 0.005), an effect driven by the reduced NAS in HFm/C vs HF/C animals (FE -1.8 ± 0.7 points, P = 0.01) in planned comparison tests (no significant difference between HFm/HF and HF/HF animals). Interestingly, Cm/HF offspring had a higher NAS than C/HF offspring in planned comparison tests (1.8 ± 0.7 points, P = 0.016).

The inflammation subscore (Figure 22, Table 18) was similarly increased by maternal and postnatal HF diets (FE +1.0 ± 0.29 points, P = 0.001; and FE 1.3 ± 0.2 points, P < 0.0001, respectively). Although the broad effect of maternal metformin treatment in HF dams reducing offspring lobular inflammation was not significant, the difference between HFm/C and HF/C offspring was significant in planned comparison tests (FE -0.8 ± 0.3, P = 0.023). The combined effect of maternal and postnatal HF diets did not exacerbate inflammation. Hepatocyte ballooning (Figure 22) was increased significantly only by the independent effect of postnatal HF diet (FE 1.1 ± 0.1 points, P < 0.0001). Steatosis, as graded qualitatively as part of the NAS (Figure 22, Table 18), was also only increased by postnatal HF diet (FE 1.4 ± 0.2 points, P < 0.0001).

Dependent variable	Significant effects	Fixed effect est.	P
Female total NAS	Maternal HF diet	1.9 ± 0.7 points	= 0.007
	Postnatal HF diet	2.4 ± 0.5 points	< 0.0001
	Metformin * Maternal HF diet	-2.2 ± 0.7 points	= 0.005
Female NAS inflammation subscore	Maternal HF diet	1.0 ± 0.29 points	= 0.001
	Postnatal HF diet	1.3 ± 0.2 points	< 0.0001
Female NAS ballooning subscore	Postnatal HF diet	1.1 ± 0.1 points	< 0.0001
Female NAS steatosis subscore	Postnatal HF diet	1.4 ± 0.2 points	< 0.0001

Table 18 | Statistical results in Figure 22. HF, high-fat; NAS, NAFLD activity score.

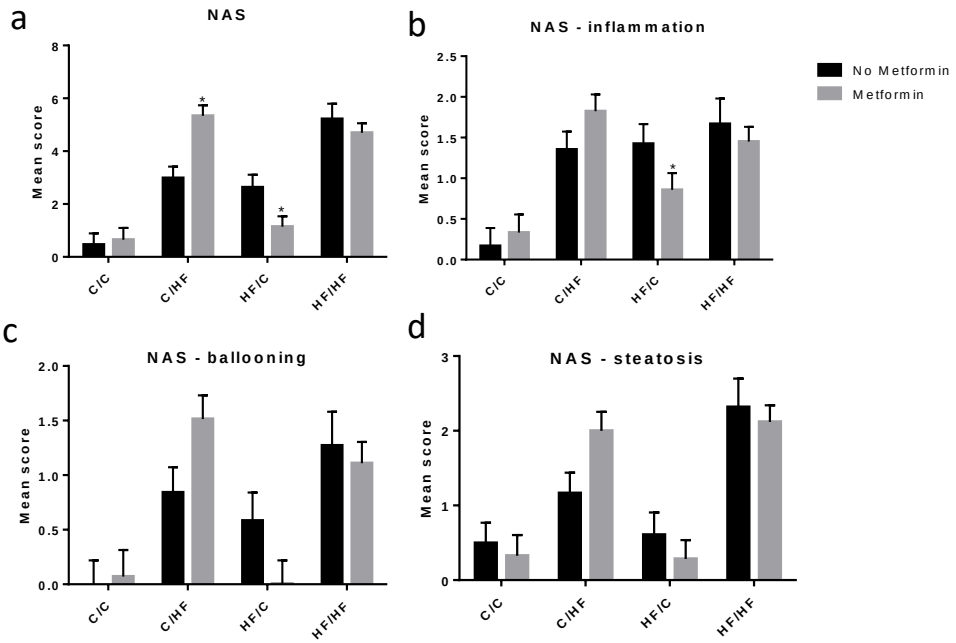

Figure 22 | **Adult female NAFLD activity score and individual domain subscores. a.** Total NAS. **b.** NAS inflammation subscore. **c**. NAS ballooning subscore. **d**. NAS steatosis subscore. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective untreated diet group in planned comparison tests**.** C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). C, chow; HF, high-fat.

Steatosis was then assessed quantitatively via point counting to determine proportion of examined hepatic area affected (Figure 21, Table 19). Here, neither postnatal nor maternal HF diets independently increased the degree of steatosis. However, combined exposure (in HF/HF and HFm/HF offspring) dramatically worsened steatosis (FE 16.9 ± 4.8%, P < 0.001). There was no effect of metformin in HF dams on offspring steatosis, but metformin in C dams when offspring

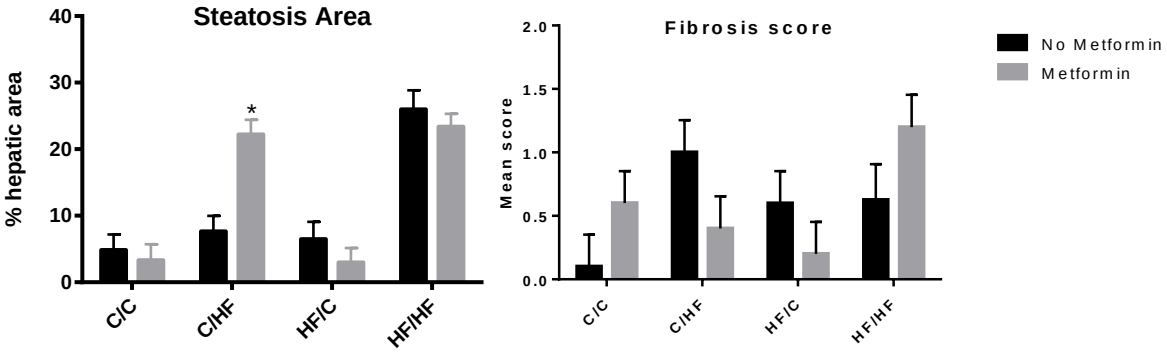

Figure 21 | **Quantitatively assessed steatosis (left panel) and qualitatively assessed fibrosis (right panel) in adult female offspring liver.** Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective untreated diet group in planned comparison tests. Females: C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters).C, chow; HF, high-fat.

were given an HF diet (Cm/HF group) markedly exacerbated steatosis compared with C/HF offspring (FE 15.9 ± 4.3%, P < 0.001).

Dependent variable	Significant effects	Fixed effect est.	P
Female steatosis %	Maternal HF diet * Postnatal HF diet	16.9 ± 4.8 %	< 0.001
Female fibrosis (sirius red stain)	Postnatal HF diet	0.48 ± 0.19 points	= 0.024

Table 19 | Statistical results from Figure 21. HF, high-fat.

Although C57BL/6 mice do not readily develop fibrosis, sirius red stain was used to qualitatively assess fibrosis by scoring according to simplified fibrosis stage (Figure 21, Table 19. Representative images in **Figure 23**). Postnatal HF diet significantly exacerbated fibrosis (FE 0.48 ± 0.19 points, P = 0.024), yet there were no other significant effects.

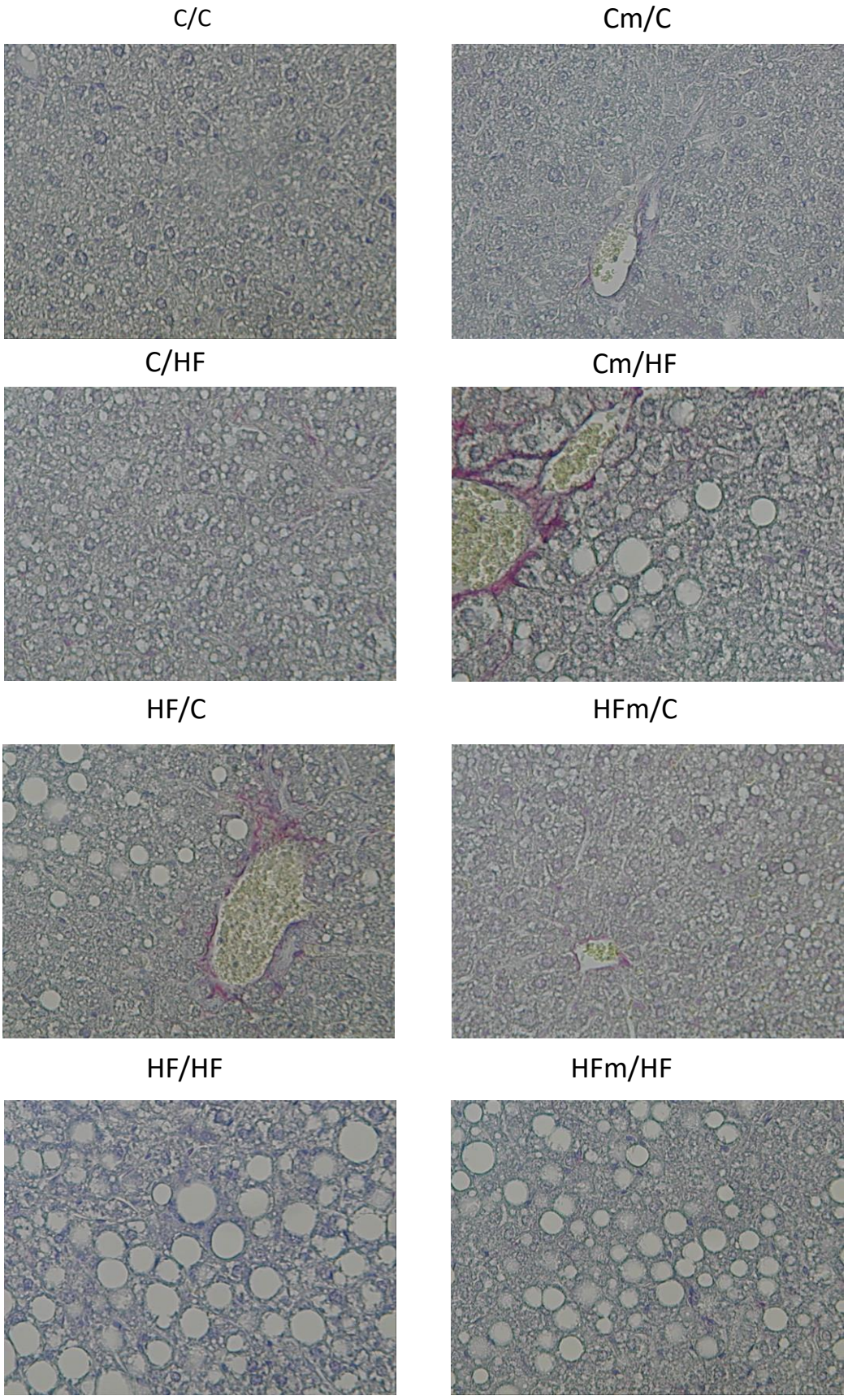

Figure 23 **| Representative images showing Sirius red staining of adult female offspring liver.** Images are representative of typical liver histology within each offspring group. Moderate periportal fibrosis is visible in Cm/HF and HF/C images (purple stain). Magnification 200x. Total number of liver assessed per group: C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). C, chow; HF, high-fat.

6.3.3 Histological assessment of NAFLD in adult male offspring

Liver histology was prepared in adult male offspring to assess NAFLD (Figure 24). In adult male offspring, NAS was increased by postnatal HF diet (FE 1.1 ± 0.5 points, P = 0.031) (Figure 25, Table 20). There was a significant interaction between maternal and postnatal HF diet, such that NAS was increased in offspring exposed to both, beyond the independent effects of maternal and

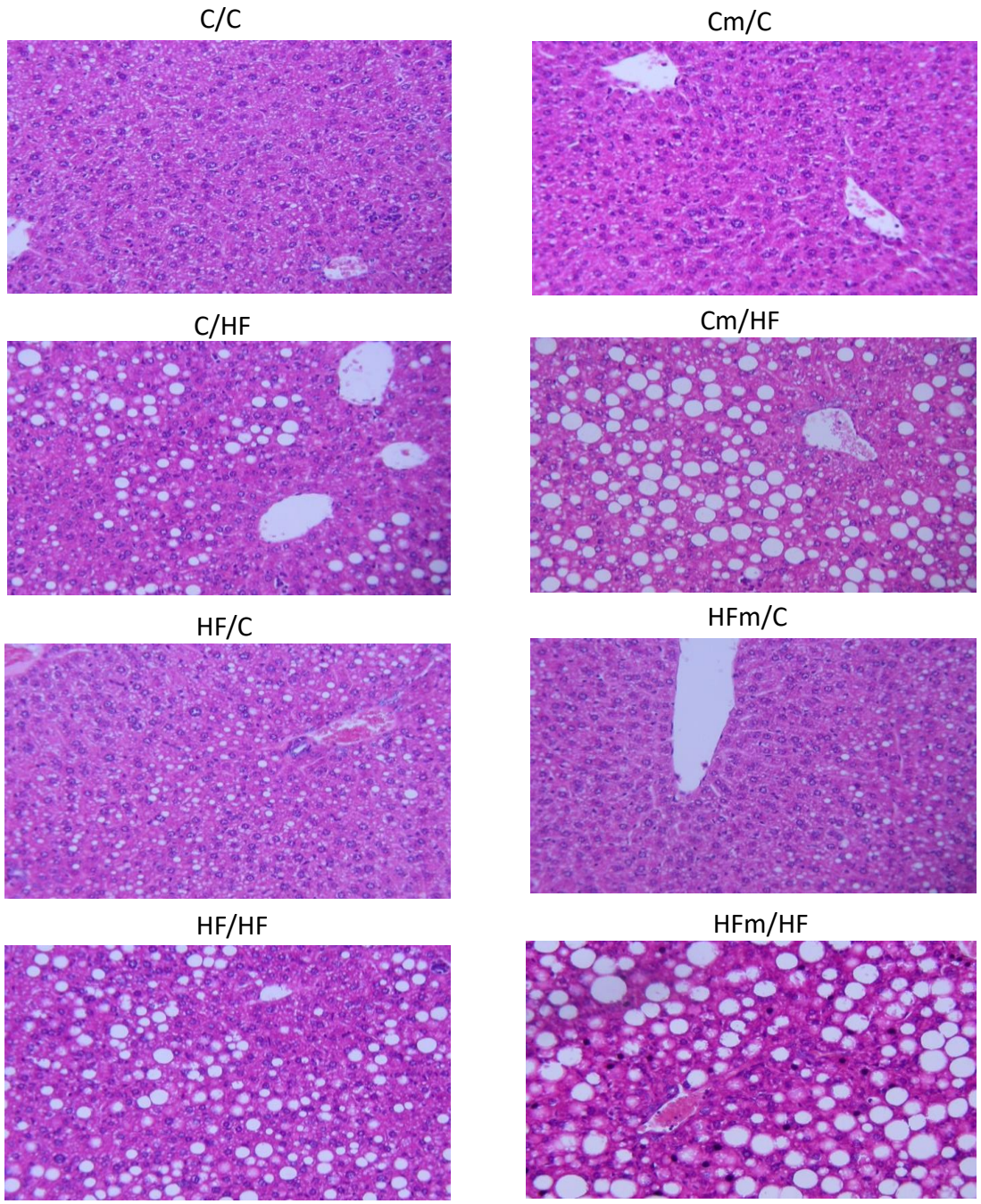

Figure 24 | **Haemotoxylin and eosin stained liver sections from adult male offspring**. Images are representative of typical liver pathology within each group. C/C and Cm/C sections show normal liver pathology. HFm/C shows mild steatosis, as does HF/C. HF/HF and HFm/HF show marked steatosis. all images taken at 200x magnification. Total number of livers assessed per group for NAS: C/C, n = 5 (from 3 litters); Cm/C, n =7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat.

postnatal HF diets (FE 2.3 ± 0.7 points, P = 0.002). As in females, Cm/HF animals had markedly more severe NAFLD than C/HF offspring (FE 2.8 ± 1.1 points, P = 0.02 in planned comparison tests).

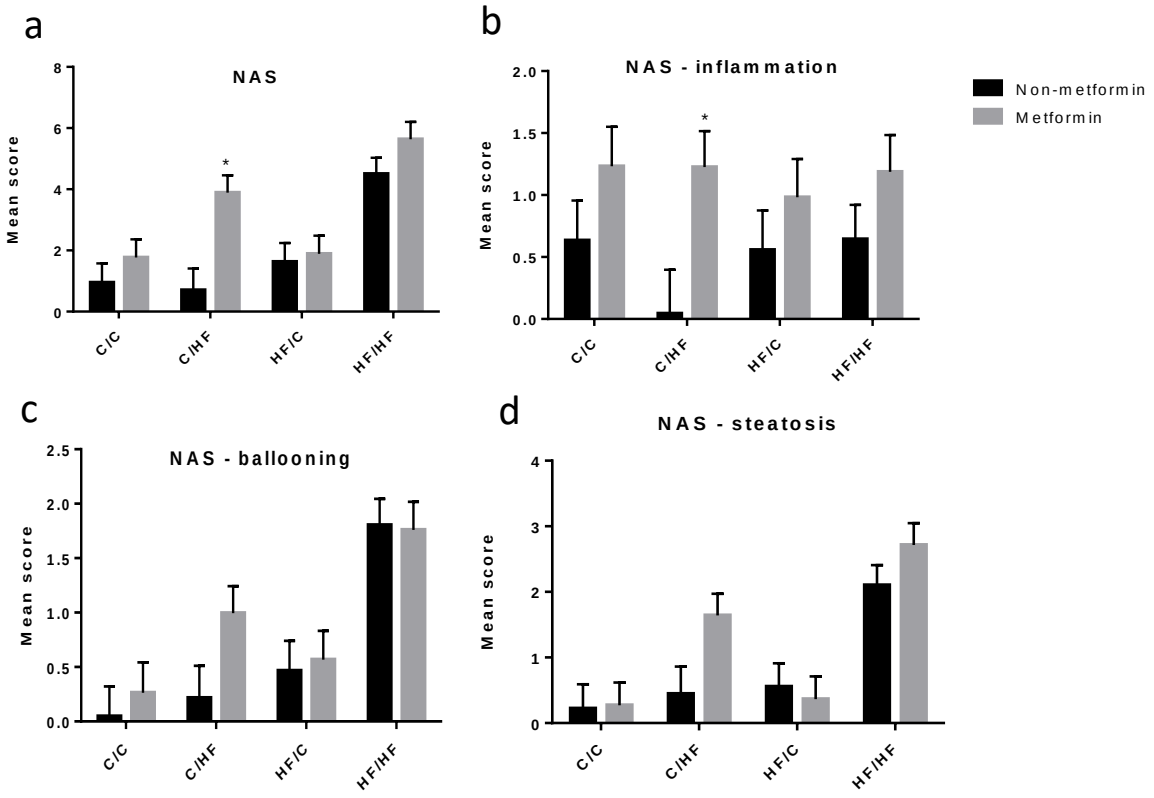

Figure 25 | **Adult male NAFLD activity score and individual domain subscores. a.** Total NAS. **b.** NAS inflammation subscore. **c**. NAS ballooning subscore. **d**. NAS steatosis subscore. Data is presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective untreated diet group in planned comparison tests. C/C, n = 5 (from 3 litters); Cm/C, n =7 (from 5 litters); C/HF, n = 6 (from 2 litters); Cm/HF, n = 8 (from 4 litters); HF/C, n = 6 (from 4 litters); HFm/C, n = 6 (from 3 litters); HF/HF, n = 7 (from 4 litters); HFm/HF, n = 7 (from 4 litters). C, chow; HF, high-fat; NAS, NAFLD activity score.

Lobular inflammation subscore (Figure 25, Table 20), in distinct contrast to female offspring, was only significantly increased by metformin treatment, irrespective of maternal or postnatal diet (FE 0.6 ± 0.2 points, P = 0.034). In planned comparison tests the increased inflammation in Cm/HF vs C/HF offspring was significant (FE 1.5 ± 0.6 points, P = 0.016). Hepatocyte ballooning (Figure 25, Table 20) was exacerbated by the independent effect of postnatal HF diet (FE 0.5 ± 0.2 points, P = 0.042), and by a combination of maternal and postnatal HF diets (interaction FE 0.8 ± 0.3 points, P = 0.027), although the independent effect of maternal HF diet did not significantly increase the degree of ballooning (P = 0.312). Steatosis severity (Figure 25, Table 20) was increased in by postnatal HF diet (FE 0.9 ± 0.3 points, P = 0.002) and again exacerbated in offspring exposed to both maternal and postnatal HF diets (FE 1.2 ± 0.4 points, P = 0.004); again, the independent effect of maternal HF diet had no effect on degree of offspring hepatic fat accumulation.

Dependent variable	Significant effects	Fixed effect est.	P
Male total NAS	Postnatal HF diet	1.1 ± 0.5 points	= 0.031
	Maternal HF diet * Postnatal HF diet	2.3 ± 0.7 points	= 0.002
Male NAS inflammation subscore	Metformin	0.6 ± 0.2 points	= 0.034
Male NAS ballooning subscore	Postnatal HF diet	0.5 ± 0.2 points	= 0.042
	Maternal HF diet * Postnatal HF diet	0.8 ± 0.3 points	= 0.027
Male NAS steatosis subscore	Postnatal HF diet	0.9 ± 0.3 points	= 0.002
	Maternal HF diet * Postnatal HF diet	1.2 ± 0.4 points	= 0.004

Table 20 | Statistical results for Figure 25. HF, high-fat; NAS, NAFLD activity score.

6.3.4 Expression on pro-inflammatory genes in adult female offspring

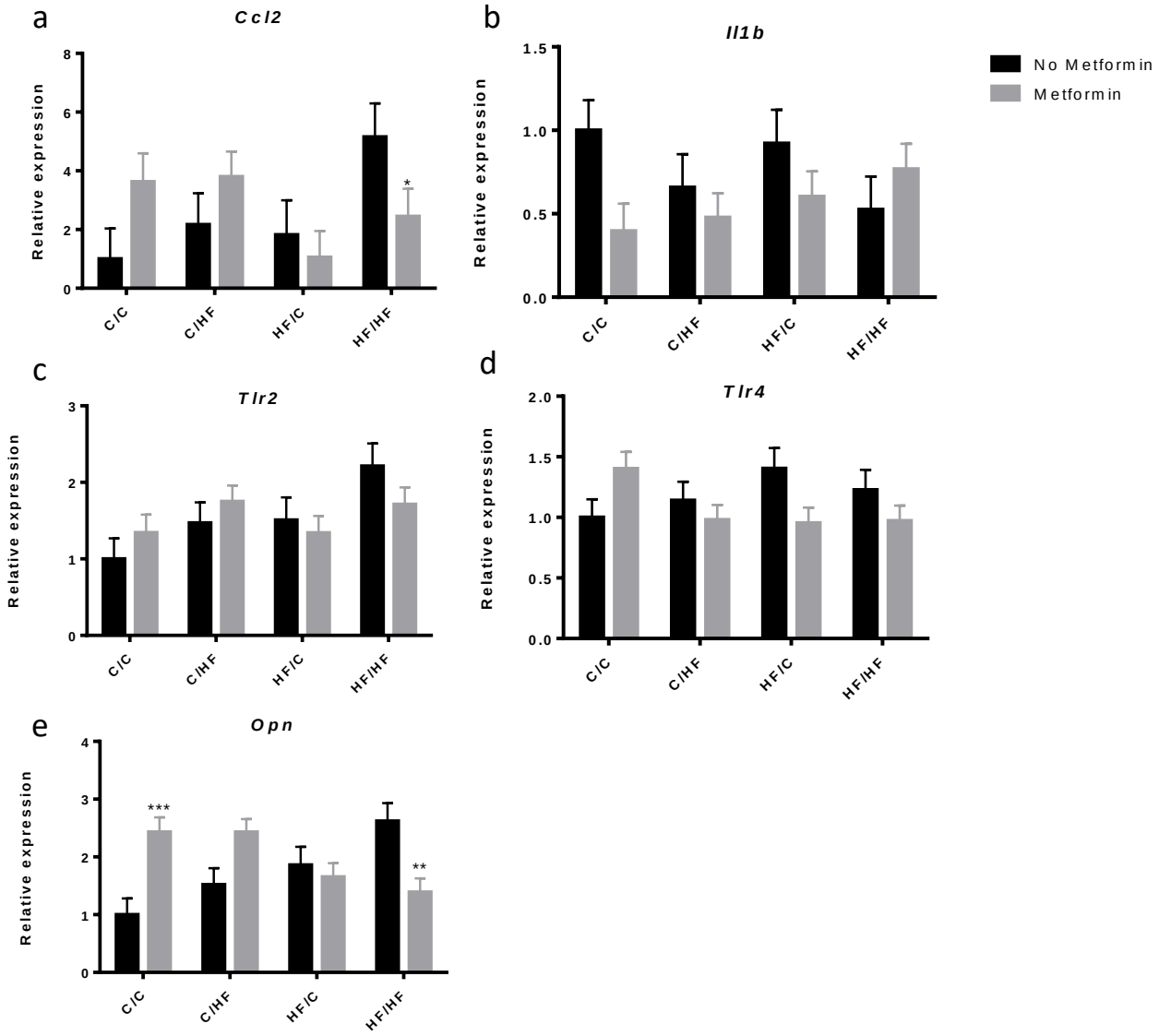

Figure 26 | **Liver expression of pro-inflammatory genes in adult female offspring.** Data is presented as predicted means ± SEM from maximal models on non-logged original data. All statistical analyses performed on log-transformed data. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01' ***, P < 0.001; ****, P < 0.0001 vs respective untreated diet group in planned comparison tests C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). C, chow; HF, high-fat.

In addition to assessing female offspring hepatic inflammation from histology, expression of genes encoding key pro-inflammatory proteins was assessed (Figure 26, Table 21). mRNA levels of *Ccl2* (also known as *Mcp1*, a gene encoding a pro-inflammatory chemotactic protein that recruits monocytes and basophils[386]) were increased by postnatal HF diet (FE 0.40 ± 0.08 log arbitrary

units (AU), P < 0.0001) and by maternal HF diet (FE 0.30 ± 0.13 log AU, P = 0.042). Maternal metformin treatment in HF dams decreased mRNA levels in offspring (FE -0.54 ± 0.17 log AU, P = 0.009). Levels of *Il1b*, encoding a pro-inflammatory cytokine, were not significantly altered in any group. Levels of the Toll-like receptor family member *Tlr2* were increased by maternal HF diet (FE 0.17 ± 0.08 log AU, P = 0.032) and to a lesser extent by postnatal HF diet (FE 0.12 ± 0.05 log AU, P = 0.014). Maternal metformin treatment in HF dams reduced expression (FE -0.21 ± 0.10 log AU, P = 0.038). *Tlr4* levels were unaltered across all groups. Next, levels of *Opn*, encoding osteopontin, which acts both as a pro-fibrotic molecule and as a pro-inflammatory cytokine, were assessed. Maternal HF diet markedly increased expression (FE 0.23 ± 0.07 log AU, P = 0.004), as did maternal metformin treatment in C-dams (FE 0.26 ± 0.07 log AU, P = 0.005). However, metformin treatment in HF-dams reduced *Opn* expression in offspring (FE -0.42 ± 0.09 log AU, P < 0.0001), an effect shown in planned comparison tests to be due to decreased expression primarily in HFm/HF vs HF/HF animals (FE -0.27 ± 0.08 log AU, P = 0.003).

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Ccl2	Maternal HF diet Postnatal HF diet Metformin * Maternal HF diet	0.40 ± 0.08 log AU 0.30 ± 0.13 log AU -0.54 ± 0.17 log AU	< 0.0001 = 0.042 = 0.009
Il1b	NONE	NA	NA
Tlr2	Maternal HF diet Postnatal HF diet Metformin * Maternal HF diet	0.17 ± 0.08 log AU 0.12 ± 0.05 log AU -0.21 ± 0.10 log AU	= 0.032 = 0.014 = 0.038
Tlr4	NONE	NA	NA
Opn	Maternal HF diet Metformin Metformin * Maternal HF diet	0.23 ± 0.07 log AU 0.26 ± 0.07 log AU -0.42 ± 0.09 log AU	= 0.004 = 0.005 < 0.0001

Table 21 | Statistical results for Figure 26. AU, arbitrary units; HF, high-fat; NA, not applicable.

6.4 Discussion

NAFLD is a chronic disease of growing societal and personal cost. Despite extensive efforts, effective pharmaceutical treatments are lacking, making prevention of the disease and mitigation of its progression important research goals. The role of maternal obesity in promoting offspring NAFLD, by both liver-specific and whole-body means, is now well-appreciated, even if the underlying mechanisms await full characterization[66]. However, the data reported here represent the first comprehensive characterisation in mice of adult offspring NAFLD severity after maternal metformin supplementation, a drug recommended for treatment of GDM by NICE in the UK and investigated for a potential therapeutic role in obese pregnancy. Specifically, the data identify effects of maternal metformin treatment that are dependent on maternal diet and/or adiposity and offspring sex. Female offspring of HF-fed dams show increased NAFLD severity as assessed by gold-standard hepatic histopathological scoring (NAS, also known as Kleiner scoring); maternal metformin treatment reduced severity substantially. However, metformin had no effect in male offspring in the same groups. In both sexes, maternal metformin treatment in lean dams markedly exacerbated the detrimental effect of postnatal HF diet. In females, Cm/HF offspring NAS was on average 1.8 points higher than C/HF offspring. In males, Cm/HF mice NAS was 2.8 points higher than the respective untreated C/HF group.

As in previous animal models of maternal developmental programming, the data presented here show that maternal HF diet, independent of postnatal HF diet, can exacerbate NAFLD in offspring. Maternal HF diet significantly increased liver weight in both male and female offspring – in females, the independent effect of maternal diet increased liver weight, whereas in males the combination of maternal and postnatal HF diet was required. Interestingly, postnatal HF diet did not independently affect liver weight in either sex, demonstrating the relevance of maternal effects compared with postnatal effects in this organ. In female offspring of HF-dams given metformin (HFm/C and HFm/HF groups), the increased liver weight seen in offspring of untreated HF-dams was almost entirely protected against, yet no effect was seen in males. In this study, the female Cm/HF group (but not the male Cm/HF group) had significantly larger livers than the respective C/HF group; increased liver weight in offspring of Cm dams in response to postnatal HF diet was reported in both males and females by Salomaki *et al*.[253]. Raw liver weight is however a nonspecific marker of NAFLD. Adjustment for body weight is sensitive to marked body weight changes and is inappropriate as a control in studies in which the experimental intervention alters body weight[387]

To accurately assess NAFLD severity, livers were scored using NAS, a validated scoring system for the assessment of NAFLD disease activity in humans and employed in animal models[269,388]. This

approach has shown in other animal models of maternal obesity that offspring of obese dams of either sex have increased disease severity, including greater steatosis, lobular inflammation and hepatocyte ballooning[70,286,382]. Data presented here mirror previously published findings, showing an effect of maternal HF diet in worsening NAFLD disease severity in both female and male offspring. In females, mean NAS was similar in both C/HF and HF/C groups. In males, although the independent effect of maternal diet on NAS was not significant, postnatal HF diet required a maternal HF diet insult to induce NAFLD. The requirement for both maternal and postnatal HF diets to induce NAFLD in offspring was also observed in female offspring in the study by Bruce *et al.*[70], and in male offspring using a similar experimental protocol by Pruis *et al*.[382]. Postnatal and maternal HF diets combined in an additive fashion in the study of Mouralidarane *et al.*[286], and Bayol *et al.* showed also increased hepatic steatosis and hepatocyte ballooning in male and female offspring receiving maternal and postnatal HF diets compared with animals receiving only postnatal diet[389]. In our study, metformin treatment in HF dams decreased NAS in female offspring, demonstrating a substantial reduction in mean NAS from 2.6 (mild NAFLD) to 1 (normal liver) in HF/C to HFm/C animals, respectively. However, the effect in HFm/HF offspring relative to HF/HF offspring was smaller, and non-significant in planned comparison testing.

In contrast to the beneficial effect in females, there was no effect of metformin treatment in HF dams in male offspring. Interventions demonstrating a beneficial effect on histologically confirmed NAFLD in animal models of developmental priming are scarce. Heerwagen *et al.* demonstrated a beneficial effect of increased maternal n-3 PUFA levels on male offspring hepatic steatosis, yet NAS was not assessed[148]. Notably, both male and female offspring of C-dams given metformin had exacerbated NAFLD severity when they were given a postnatal HF diet. In males, mean NAS score was increased around fourfold in Cm/HF vs C/HF offspring, from no significant disease to moderate NAFLD. In females, mean NAS score in Cm/HF offspring indicated moderate NASH, whereas C/HF offspring had NAFLD. These data present, for the first time, a severe detrimental effect on offspring NAFLD disease course resulting from inappropriate maternal metformin supplementation. One can hypothesize that metformin in C-dams, in contrast to effects in HF-dams, elicits physiological responses similar to undernutrition. Maternal undernutrition models show increased markers of liver disease in offspring when exposed to an additional postnatal obesogenic diet. For instance, George *et al.*[334] showed that adult female offspring of nutrient-restricted ewes gained more weight and had an increased degree of hepatic steatosis when given a postnatal energy-rich diet postnatally, compared to offspring of non-nutrient-restricted ewes. Similar findings were reported in another sheep model of maternal nutrient restriction by Hyatt *et al.*[260], and a 2016 report by Zhang *et al.* showed increased NAS in female rats born to nutrient-restricted dams compared with those born to control dams[388].

Mechanisms underlying the increased risk of NAFLD in these offspring are unclear. In the models of Hyatt *et al.* and George *et al*., maternal nutrient restriction increased offspring obesity and decreased insulin sensitivity in response to postnatal obesogenic diet. Thus, the more severe NAFLD phenotype in these animals might be the result of increased lipid delivery to the liver from unsuppressed adipose tissue lipolysis. Conversely, in the model reported by Zhang *et al*., offspring body weight and fasting blood glucose levels were not altered by exposure to maternal nutrient restriction, yet hepatic mRNA levels of the prolipogenic transcription factor *Srebp1c* were increased in female offspring, and expression of *Cpt1*, which encodes the rate-limiting enzyme in fatty acid β-oxidation, was decreased. In this chapter, both male and female Cm offspring had glucose intolerance and increased levels of body fat, although only males had significant hyperinsulinaemia and increased insulin resistance. Increased lipolysis could therefore contribute to the increased levels of hepatic steatosis in both males and females, yet other factors, such as impaired hepatic fatty acid oxidation or increased DNL, might also contribute. These effects are investigated in Chapter 7.

Analysis of the individual components of the NAS in females did not reveal significant divergence in any individual components from the sum NAS score; that is, the relative scores in the inflammation, hepatocyte ballooning and steatosis domains followed the pattern of the total NAS score. These findings suggest that individual experimental groups do not experience priming or exacerbation of one particular facet of NAFLD. However, in males, despite the degree of steatosis and hepatocyte ballooning mirroring the total NAS, the degree of lobular inflammation was exacerbated by metformin treatment regardless of maternal or postnatal HF diet. Whether metformin exerts this effect by a uniform mechanism across all male offspring groups is unclear, but the fact that the inflammation subscore seems independent of changes in physiology tentatively suggests this might the case. Alteration of early-life microbiota colonization could be one potential mechanism by which metformin alters offspring hepatic inflammatory phenotype irrespective of maternal and postnatal diet. Mice treated with metformin have altered gut microbial composition by acute exposure, and altered transfer of the gut microbiota during early-life, leading to differences in gut microbiota composition, might have marked persistent effects on offspring metabolism[390] and specifically NAFLD risk via increased gut permeability and hepatic exposure to bacterial products that promote inflammation[391]. Offspring of moderately overweight female mice given metformin during pregnancy had altered gut microbiota composition compared with offspring of dams not given metformin[392]. However, whether these changes induced by metformin are then associated with increased hepatic inflammation have not been investigated.

On the basis that the qualitative assessment of steatosis (as a part of the NAS) in females showed a significant difference resulting from metformin treatment, female offspring steatosis was assessed semi-quantitatively. These measurements showed that maternal and postnatal HF diets did not independently induce hepatosteatosis, yet a combination of both exposures increased lipid accumulation, in line with the findings of Bayol *et al.*[389]. Metformin did not reduce steatosis in offspring of HF-dams, but steatosis was markedly exacerbated in Cm/HF vs C/HF offspring, confirming the broad effects of treatment in the liver of lean offspring exposed to a postnatal HF diet. Although C57BL/6 mice do not readily develop hepatic fibrosis as a result of dietary challenge[273], this parameter was assessed in female offspring by staining tissue sections with Sirius red stain. Here, only postnatal HF diet significantly increased fibrosis, although fibrosis scores were mild in comparison to human NASH across all groups.

Molecular markers of inflammation in female offspring livers were measured, as a result of the significantly altered NAS inflammation subscore in HFm/C offspring. Supporting the role of maternal diet in increasing hepatic inflammation, mRNA levels of the pro-inflammatory chemokines *Ccl2* and *Opn* were increased independently by maternal HF diet. *Ccl2*, encoding a ligand for C-C chemokine receptor 2, is increased in humans with NASH and in animal models of the disease [393]. *Ccl2* levels have also been shown to be increased in male offspring by maternal obesogenic diet in animals, yet only when offspring were also given a postnatal HF diet [382]. Similar results were observed in this study in females, indicating that increased *Ccl2* expression is primed by maternal HF diet, but requires a postnatal dietary insult to manifest. Metformin significantly reduced *Ccl2* levels, with the reduction in HFm/HF vs HF/HF offspring responsible for most of the apparent effect.

Tlr2 and *Tlr4* are members of the Toll-like receptor family of transmembrane proteins recognizing pathogen-associated molecular patterns (PAMPs) and endogenous molecules derived from damaged host cells, termed damage-associated molecular patterns (DAMPs)[394]. The role of TLR4 in NASH is well-appreciated; deficiency of TLR4 in mice lessens NASH and fibrosis severity[395], and hepatic *TLR4* mRNA expression is increased in patients with NASH but not in patients with NAFLD[396]. Although levels of *Tlr4* were not altered in the data reported in this thesis, levels of *Tlr2* were significantly increased independently by maternal and postnatal HF diets. *Tlr2*-knockout mice are markedly protected against diet-induced adiposity and hepatic steatosis[397] and hepatic fibrosis[398]. Thus, reduced expression of *Tlr2* in this model might underlie altered hepatic inflammation between metformin and non-metformin-exposed female offspring, although the magnitude of the treatment-induced expression change is relatively small. Given that Kupffer cells are the predominant liver cell type expressing *Tlr2*[399], and Kupffer cell *Tlr2* expression is required for *Tlr2*-specific pro-inflammatory and hepatosteatotic effects[398], analysis of Kupffer-cell-specific

Tlr2 expression is required to properly characterize the role of this pathway in this model. However, hepatocytes also upregulate *Tlr2* expression in response to LPS and key pro-inflammatory molecules, including TNF[394]. Thus, metformin might alter *Tlr2* expression via direct innate immune priming mechanisms, or by reducing levels of signalling molecules inducing *Tlr2* expression; for instance, the integrity of the gut epithelium is not assessed in this model, and priming of a 'leaky gut' by maternal obesity (evidence of which is reviewed elsewhere[400]) could increase intrahepatic levels of bacteria-derived TLR2 ligands such as LPS.

OPN plays a critical role in fibrogenesis. Patients with NASH have increased hepatic levels of OPN, and OPN deficiency in mouse models of liver fibrosis decreases fibrosis severity[401]. Moreover, neutralisation of OPN *in vivo* in mice protected against development of liver fibrosis in three different models[402]. The data presented here of a marked reduction in *Opn* expression in HFm/HF vs HF/HF animals is therefore a promising beneficial outcome of metformin treatment, given the significant effect of maternal HF diet on increasing offspring *Opn* levels. We did not observe an effect on histologically assessed fibrosis, suggesting that the increased expression of OPN is an early event in fibrosis, which is suppressed by metformin.

Metformin treatment in lean dams significantly increased offspring hepatic *Opn* expression, with the majority of the detrimental effect observed in Cm/C vs C/C offspring. Thus, metformin in the absence of maternal obesity seems to prime increased *Opn* expression independent of postnatal HF diet, and might represent accelerated fibrosis in these animals. Interestingly, Tarry-Adkins *et al.* showed that male offspring in a rat maternal protein restriction model had increased hepatic fibrosis and inflammation, demonstrating the detriment effect of early-life undernutrition on NASH development. As outlined in Chapter 4, metformin in lean dams might promote similar responses in lean dams as caloric or protein restriction.

Despite other groups showing altered offspring levels of hepatic *Tnf*[287,382] as a result of maternal obesity, no significant expression was detected in the animals used in this thesis. Diet and model differences notwithstanding, the PCR experiments of Oben and Pruis used the housekeeping genes *Gapdh* and *36b4*, respectively, to normalize expression. Although the stability of *36b4* in murine maternal obesity models is unknown, *Gapdh* expression has been shown to be unstable in the model employed in this study, leading to the selection of *Ywhaz* as the housekeeping gene to normalize the PCR data reported here[282]. These data highlight the importance of appropriate control selection and, in addition to differences in experimental protocols, might underpin contrasting gene expression results between studies.

In summary, these data show for the first time that maternal metformin treatment ameliorates some of the maternal-obesity-primed increase in NAFLD severity, albeit only in female offspring.

HFm/C females showed a reduced total NAS, although this was not associated with reductions in levels of pro-inflammatory markers. This lack of metformin effect on offspring molecular markers, despite an apparent reduction in NAS inflammation subscore, might be the result of metformin acting on alternative pathways involved in leukocyte recruitment in sterile liver injury[403]. Although CCL2 is a primary chemokine in this setting, other molecules are also involved; for instance, CCR8 is required for hepatic macrophage recruitment in two mouse sterile liver injury models[404]. Alternatively, the relatively low levels of infiltrating immune cells seen in mice with NASH in this model, compared with human NASH, might mask any alteration in immune cell pro-inflammatory gene expression[405]. Quantitative assessment of immune-cell infiltration, or characterization of the activation states and gene expression of these cells, was not performed in this study but should be considered as a potential next step in assessing the effects of metformin and to verify the authenticity of the current findings. In addition to not affecting molecular markers of inflammation, there was no apparent effect of maternal metformin in HFm/C animals on degree of steatosis, suggesting that inflammation differences in this group compared with HF/C offspring are independent of changes in total steatosis. As lipid metabolism and lipid composition might also be altered in this model by metformin and maternal obesity, Chapter 7 explores the hepatic lipid makeup.

In HFm/HF offspring, metformin did not reduce NAS compared with HF/HF offspring, and no individual subscore components were significantly reduced, suggesting that postnatal HF diet overwhelms the protective effect of metformin in obese dams. Despite this finding, the chemokines *Opn* and *Ccl2* were reduced substantially. One would expect reduced levels of *Ccl2* to be associated with reduced leukocyte recruitment. Other chemokines might be responsible for the immune infiltrate, and high levels of their expression might mask the beneficial effect of reduced *Ccl2* expression[403]. Alternatively, the low sensitivity of the subjective, qualitative assessment of lobular inflammation might mask any statistically significant reduction between HFm/HF and HF/HF animals. In the absence of marked immune cell infiltrates and development of fibrosis in this model relative to human NASH, molecular markers of inflammation take on greater importance, and subsequent work to better characterize the immune and profibrotic response is required.

The most pertinent results presented in this Chapter are that offspring of lean dams are at increased risk of more severe NAFLD, and that markers of inflammation are increased even in those offspring not receiving a postnatal HF diet. The increased NAFLD severity is, unlike the beneficial effect of metformin, independent of sex. Most notably, total NAS, lobular inflammation subscore, steatosis subscore (and quantitative assessment of steatosis), hepatocyte ballooning subscore were all significantly or trended higher in Cm/HF offspring than C/HF offspring, and

female offspring also showed significantly increased or trends in the increase of pro-inflammatory molecular markers. Together, these data strongly suggest that inappropriate metformin treatment in lean dams has dramatic effects on offspring response to a postnatal HF diet challenge. Salomaki *et al*. showed that prenatal metformin in lean mice programmed risk of a NAFLD-associated phenotype (impaired glucose homeostasis, increased adiposity and enlarged livers) after postnatal HF diet[253]. However, they did not explore the adult offspring liver phenotype beyond liver weights, despite using their data to urge caution. The data reported in this chapter, using a similar mouse model, confirms the persistence of metformin-mediated priming effects on NAFLD risk in adult offspring.

Chapter 7: Maternal metformin alters offspring hepatic fatty acid composition

7.1 Introduction

Hepatic lipid accumulation is central to the pathogenesis of NAFLD. Beyond excess total lipid, data show that hepatic fatty acid composition is also altered in patients with NAFLD, and such changes might alter disease phenotype and severity or be representative of altered hepatic lipid metabolism. Lipidomic analyses of liver samples from patients with steatosis without marked inflammation (so-called 'simple steatosis', or NAFL), NAFLD or NASH have revealed differences in hepatic lipid composition as severity of NAFLD increases[44]. As individual fatty acids and their storage forms can exert specific physiological effects, both positive and negative in the context of metabolism and inflammation, this composition is of interest in NAFLD. Moreover, alterations in the absolute or relative abundances of individual lipid species can reflect activity of synthetic or catabolic pathways altered according to disease state, providing a biomarker for the activity of potentially pathogenic mechanisms[406].

7.1.1 Fatty acids

Fatty acids can be considered the basic component of lipids, and in animal tissues are found most commonly as a straight (rather than branched) chain of an even number of carbon atoms, with a carboxylic acid group at the head and a terminal methyl group. Palmitic acid (palmitate), a fatty acid of 16 carbons length with no unsaturated double bonds (hence designated 16:0) is the most common saturated fatty acid in animals[407]. When not bound via an ester bond to another molecule (such as glycerol, to form glycerides, or cholesterol, to form cholesterol esters (CEs)), fatty acids can be described as nonesterified, or free, fatty acids. Common examples of individual fatty acid species are given in Figure 27. Monounsaturated fatty acids (MUFAs) contain one double bond at some point along their length. For instance, oleic acid, the most abundant MUFA in nature, contains a double bound at the 9th carbon from terminal methyl group, and is designated in short-hand as 18:1(n-9). Polyunsaturated fatty acids (PUFAs) contain multiple double bonds; alpha-linolenic acid (ALA), for example, contains 3 double bonds starting 3 carbons from the terminal methyl group, and is 18 carbons in length, thus being given the designation 18:3(n-3). By contrast, n-6 PUFAs contain the first double bond 6 carbons from the terminal methyl group; linoleic acid contains two double bond, is 18 carbons long and is thus given the designation 18:2(n-6).

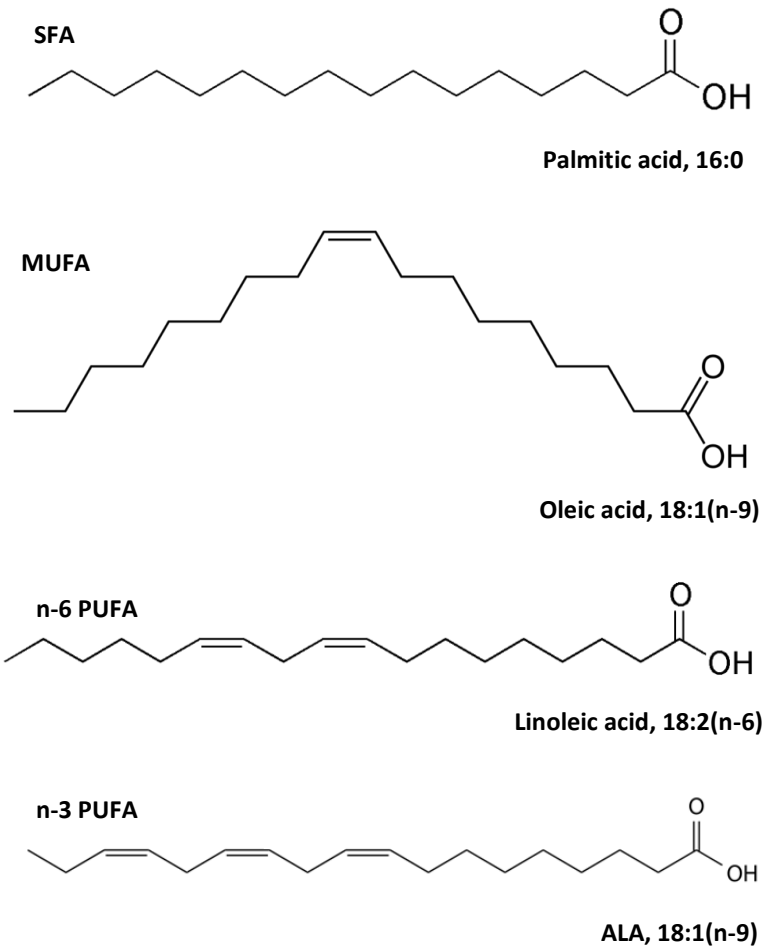

Figure 27 | **Common examples of long-chain fatty acids found in mammals.** Common names and short hand names are provided, with short hand names describing the position and number of the double bonds, in addition to the chain length. ALA, alpha-linolenic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. Image structures are public domain.

Mammals are unable to synthesize linoleic acid nor ALA, which can be synthesized in plants by desaturation of linoleic acid; as a result, linoleic acid and ALA are considered essential fatty acids in mammals, which must be obtained from dietary sources in order to allow synthesis of other fatty acid species[407]. Linoleic acid can be desaturated (via desaturases) and elongated (via elongases) to form other n-6 PUFAs, including arachidonic acid (20:4(n-6)), which is a precursor for production of lipid signalling molecules. Similarly, ALA can be metabolized to eicosapentaenoic acid (EPA; 20:5(n-3)) and docosohexaenoic acid (DHA (22:6(n-3)), although conversion of EPA to DHA (via docosapentaenoic acid) is limited in humans (estimated at ~1% of ingested ALA)[408], making dietary sources the predominant source of DHA[409]. As the same group of enzymes performs the conversion of ALA and linoleic acid to EPA/DHA and arachidonic acid, respectively,

excess consumption of linoleic acid can saturate and impair the production of EPA and DHA. Thus, the ratio of ingested n-3:n-6 PUFAs is important for efficient production of EPA and DHA[409].

7.1.1.1 PUFAs

PUFAs have attracted notable attention for their bioactive properties. The n-3 PUFAs EPA and DHA in particular have been investigated extensively for their anti-inflammatory and lipid-metabolism-regulating properties. n-3 PUFAs are strong activators of peroxisome proliferator-activated receptors (PPARs), and activation of PPARα in the liver upregulates β-oxidation of fatty acids[410]. Moreover, increased dietary levels of DHA and EPA also suppress expression of HF-diet-induced hepatic prolipogenic genes in mice[411]. In addition to substantial roles in regulating lipid metabolism, n-3 PUFAs are also required for the synthesis of resolvins and protectins, metabolites that exert potent anti-inflammatory and inflammation-resolving effects[412].

n-6 PUFAs are also involved in the process of signalling molecules. However, in contrast to the products of n-3 PUFAs, downstream products of the n-6 PUFA arachidonic acid, such as series 2 prostaglandins, series 4 leukotrienes and lipoxins, are considered pro-inflammatory[413]. In addition, inflammatory cells typically contain a high proportion of arachidonic acid relative to other cell types, providing a substrate reservoir for the synthesis of these pro-inflammatory molecules[413].

On the basis of these positive effects on metabolic health, and as diets in the West are low in n-3 PUFAs relative to n-6 PUFAs, studies have investigated supplementing diets with DHA and EPA as a treatment for NAFLD. In the Welcome study (a randomized, placebo-controlled trial), 15-18 month treatment with 4 g of combined DHA and EPA per day reduced the amount of liver fat in patients with NAFLD when data were adjusted for erythrocyte n-3 PUFA enrichment[46]. A recent meta-analysis of ten randomized controlled trials found evidence that n-3 PUFA supplementation can reduce liver fat, although the authors opined that more data are needed[414]. In another randomized placebo controlled trial, Argo *et al.* showed that although 3 g per day n-3 supplementation reduced liver fat in patients with NASH, there was no histological improvement in disease severity[415].

7.1.2 *De novo* lipogenesis

Hepatic DNL, the process by which carbohydrates are converted into fatty acids and downstream lipids, is markedly upregulated in patients with NAFLD compared with BMI-matched controls[18]. As such, DNL is considered a key process contributing to hepatic steatosis; stable-isotope labelling indicates that ~26% of hepatic lipids in patients with NAFLD originate from DNL (compared with ~60% from lipolysis of adipose tissue and ~15% from the diet)[18]. A bifurcation in the hepatic

insulin signalling pathway allows a paradoxical insulin-mediated increased in DNL, in parallel with insulin-resistance-mediated increases in gluconeogenesis[416]. mTORC1, which lies downstream of AKT, can promote lipogenic gene expression (via SREBP1c) while not inhibiting FOXO1, the primary transcription factor regulating expression of gluconeogenic genes[39].

DNL allows the storage of excess carbohydrate by converting acetyl-CoA into fatty acids and TAG (Figure 28). The key enzymes involved in the production of fatty acids, which are subsequently condensed on to a glycerol backbone to form TAG, are: ATP citrate lyase (ACLY; which synthesizes cytoplasmic acetyl-CoA from citrate leaving the citric acid cycle); acetyl-CoA carboxylase (ACC; which forms malonyl-CoA from acetyl-CoA, the rate-limiting step in DNL); and fatty acid synthase (FAS; which produces the saturated fatty acid palmitic acid from malonyl-CoA)[416]. Palmitic acid produced by DNL can then be elongated by elongase of long chain fatty acids 6 (ELOV6) to produce longer chain saturated fatty acids, such as stearic acid (18:0). In addition, palmitic acid can be desaturated by stearoyl-CoA desaturase (SCD). SCD catalyses the rate-limiting step in the production of monounsaturated fatty acids (MUFAs), primarily producing oleic acid and palmitoleic acid (16:1n-7) from the saturated precursor fatty acids (stearic acid and palmitic acid, respectively)[416].

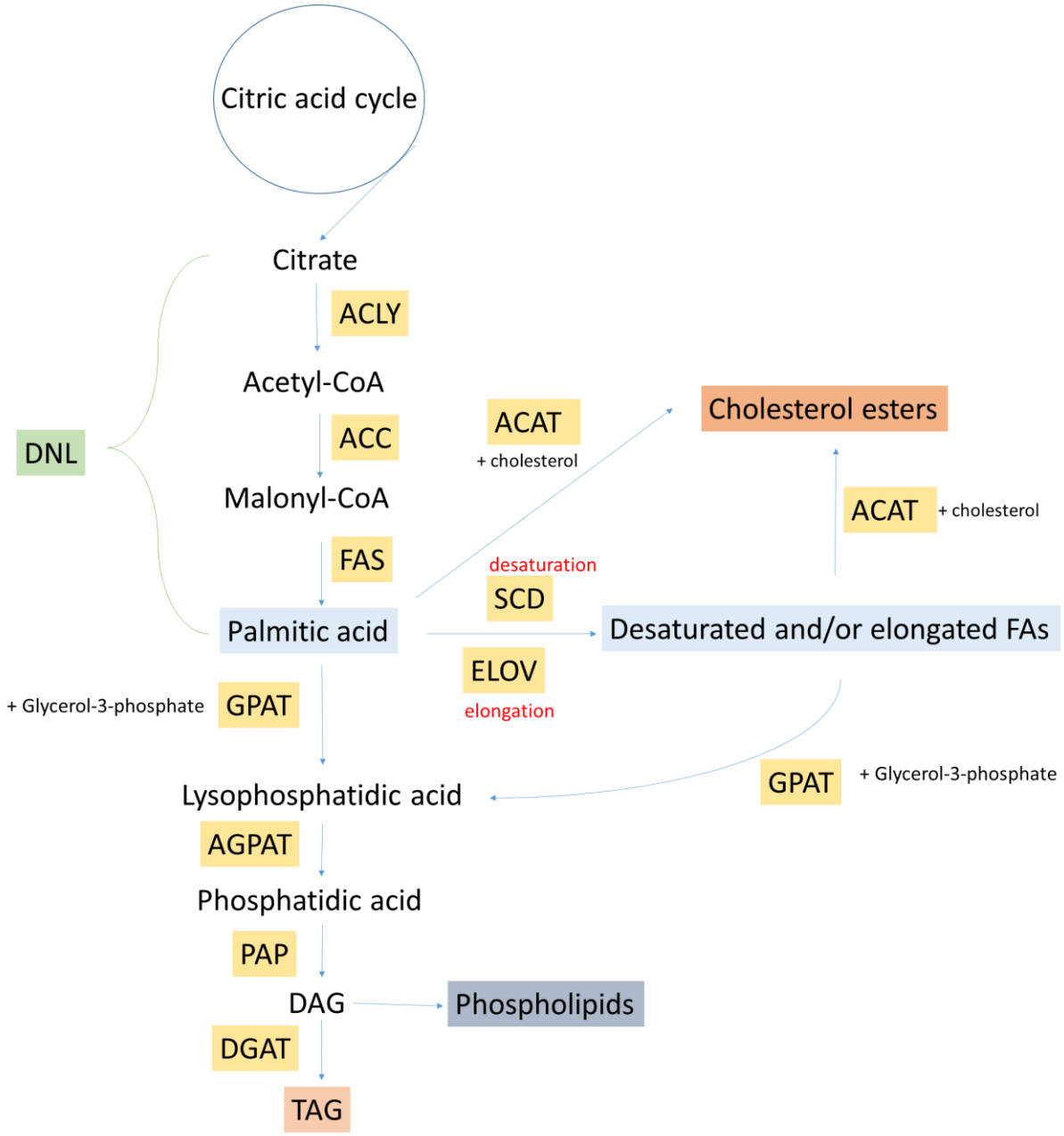

Figure 28 | **Core enzymes and metabolites involved in hepatic DNL**. Citrate, ultimately derived from cellular glucose, exits the citric acid cycle and is converted to palmitic acid by core lipogenic enzymes, ACLY, ACC and FAS. Palmitic acid can then undergo elongation and/or desaturation to form other fatty acid species. TAG is formed from individual fatty acids in a process catalysed by AGPAT, PAP and DGAT. ACC, acetyl-CoA carboxylase; ACAT, acyl-coenzyme A:cholesterol acyltransferase; ACLY, ATP citrate lyase; AGPAT, acylglycerol-3-phosphate O-acyltransferase; DAG, diacylglyceride; DGAT, diglyceride acyltransferase; ELOV, elongation of very long-chain fatty acids; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; PAP, phosphatidate acid phosphatase; SCD, stearoyl-CoA desaturase; TAG, triglyceride.

In sum, DNL leads to increased production of palmitic acid; the ratio of palmitic acid to the n-6 monounsaturated fatty acid linoleic acid (18:2(n-6)), an essential FA not synthesized by mammals

and derived only from dietary sources or adipose reserves, provides an indication of DNL rate[417]. Moreover, activity of SCD can be inferred from the ratio of palmitoleic acid to palmitic acid[418]. These ratios are generally employed on analysed VLDL-TAG FAs in clinical settings to avoid liver biopsy, yet liver biopsy samples provide a more accurate representation of the hepatic lipid pool. The use of these ratios in plasma FFA VLDL-TAGs has been validated against gold-standard stable isotope studies in humans[419].

7.1.3 Major lipid fractions

Individual fatty acids are found in a number of lipid fractions in mammalian cells, reflecting the diverse functions of lipids. The predominant lipid classes by hepatic abundance[420], especially within lipid droplets found in patients with NAFLD[44], are: TAGs, CEs, phospholipids (of which phosphatidylcholines represent the largest class) and, to a lesser extent, nonesterified fatty acids.

7.1.3.1 TAGs

TAG provides an inert, long-term storage form in which to store fatty acids. Although healthy liver does not contain substantial amounts of TAG, and increased storage of TAG (such as in hepatosteatosis) is associated with hepatic insulin resistance, evidence suggests that an impaired ability to store excess hepatic fatty acids in hepatic TAG increases insulin resistance due to accumulation of lipotoxic lipid intermediates[421]. Therefore, effective hepatic TAG storage can be seen as a protective effect, emphasised by data showing that overexpression of DGAT, and thus increased TAG synthesis, protects against hepatic insulin resistance[48].

Individual fatty acids produced by DNL and obtained from dietary sources can be stored by their condensation onto a glycerol-3-phosphate backbone, forming TAGs, which comprise three fatty acid molecules. The first step of this process is catalysed by glycerol-3-phosphate acyltransferase (GPAT), producing lysophosphatidic acid. Phosphatidic acid is then produced by the action of acylglycerol-3-phosphate acyltransferase (AGPAT), which is then further processed to diacylglycerol (DAG) by phosphatidic acid phosphorylase (PAP). DAG species are notable for being strongly associated with hepatic insulin resistance and lipotoxicity[34]. TAGs are finally formed by the addition of the final fatty acid moiety, catalysed by diacylglycerol acyltransferase (DGAT)[422].

TAG typically represents the most abundant hepatic lipid fraction in patients with NAFLD; Puri *et al*. showed an increase from 13.6 µmol/g liver tissue in healthy BMI-matched controls to 129 µmol/g tissue in patients with NAFLD[44]. In addition, patients with NAFLD had reduced levels of PUFAs, both n-3 and n-6, in their TAG fatty acid pool, with substantial depletion of DHA and arachidonic acid, an important n-6 PUFA acting a precursor in the synthesis of eicosanoids.

7.1.3.2 Cholesterol esters

CEs are more inert than either free cholesterol or FFAs, and thus represent a storage form of both molecules; rather than contributing to membrane fluidity (as per free cholesterol), cholesterol esters are stored in lipid droplets, along with TAGs[65]. In the liver, the enzyme acyl-CoA cholesterol acyltransferase (ACAT) is responsible for the formation of CE from FFA and cholesterol. Conversely, when free cholesterol or fatty acids are required for maintenance of cellular lipid homeostasis, cholesterol ester hydrolases are able to break the cholesterol ester bond to provide both species.

Puri *et al.* found no significant increase in abundance of CEs between healthy individuals and those with either NAFLD or NASH, although they did see an increase in free cholesterol in patients with NASH compared with those with NAFLD or BMI-matched healthy individuals[44]. Notably, in contrast to the TAG fraction, there was an enriched abundance of n-3 and n-6 PUFAs in the CE fraction in patients with NAFLD or NASH.

7.1.3.3 Phosphatidylcholines

Phosphatidylcholines (PCs) are a primary component of the hepatic lipid pool, and have important functions within the cell membrane. The molecule comprises a choline head group with two fatty acid moieties, typically one saturated and one unsaturated fatty acid. Synthesis of PC can proceed via two mechanisms: firstly, via reaction of cytidine disphosphocholine with DAG; and secondly, via sequential methylation of phosphatidylethanolamine (PE). After synthesis, acyl remodelling (that is, enzyme-mediated modification of phosphatidylcholine fatty acid composition) is a continuous process[407]. A reduced ratio of PC to PE in lipid droplet membranes has been implicated in increased coalescence (fusion of lipid droplets) and progression of NAFLD[423]; moreover, humans with NAFLD typically have reduced levels of PC in their livers[44].

Changes to the composition of membrane phospholipids, in particular the relative amounts of n-3 and n-6 PUFAs, can exert changes in cellular and organ function[424]. For example, knockdown of SCD in HeLa cells increased the proportion of saturated fatty acids incorporated into membrane PCs and activated the unfolded protein response and increased endoplasmic reticulum stress[425]. Typically, PCs in the endoplasmic reticulum are highly unsaturated for increased membrane fluidity[426]. In addition, inflammatory cells typically contain a high proportion of arachidonic acid in membrane phospholipids, which serves as a reservoir of substrate for the synthesis of pro-inflammatory eicosanoids[424]. Supplementation with n-3 PUFA can reduce the level of membrane arachidonic acid, thereby potentially altering the production of downstream pro-inflammatory metabolites[427].

The effect of maternal obesity on priming of hepatic lipid composition has been reported in animal models, although the effect of metformin treatment in these models in adult offspring has not been assessed. Numerous groups have reported increased levels of hepatic TAG or total lipids in adult offspring as a result of independent exposure to maternal obesity, and exacerbated accumulation of hepatic lipids with combined maternal and postnatal HF diet[70,382]. Although human studies have documented increased hepatic lipids in neonates born to obese mothers using noninvasive screening techniques[68], and a number of studies have described lipotoxicity and/or fatty acid composition in fetal liver in maternal obesity animal models, including in nonhuman primates[74,251], few studies have characterized hepatic fatty acid composition in adult offspring. At this time point, fatty acid abundance reflects postnatal diet and priming of fatty acid metabolism pathway activities. Seet *et al.* reported in mice that the proportion of select fatty acids of interest (including palmitic, palmitoleic, linoleic and arachidonic acid) in 6-month-old male offspring of obese HF-fed dams was not altered by the independent effect of maternal HF diet[428]. However, they did find that hepatic desaturase index was increased, alongside increased SCD-1 expression. Interestingly, Pruis and colleagues characterized the lipid composition in 30-week-old offspring in their mouse model of maternal obesity[382]. Offspring livers exposed to both maternal and postnatal overnutrition had increased levels of cholesterol and TAG. Moreover, offspring exposed to both maternal obesity and postnatal overnutrition had increased relative abundance of hepatic MUFAs, and decreased relative abundance of hepatic PUFAs, compared with offspring who were only exposed to postnatal overnutrition.

Given the utility of hepatic fatty acid composition as a biomarker of the activity of key lipid metabolism pathways, and the bioactive properties of different lipid species, the hepatic fatty acid composition in four important lipid fractions (TAGs, CEs, phosphatidylcholines and NEFAs) of adult offspring was assessed using gas chromatography.

7.2 Methods

7.2.1 Animal model

Offspring exposed to combinations of maternal obesity, postnatal obesity and maternal metformin treatment were generated as described in Chapter 3. Briefly, female mice were given C (7% kcal as fat) or HF (45% kcal as fat) diets for 6 weeks prior to mating with C-fed males. At confirmation of mating, males were removed and half of the successfully mated females were given metformin (m) dissolved in drinking water (250mg/kg body weight) for the duration of pregnancy and lactation. Pups were weaned at 3 weeks onto either HF or C diets, generating 8 offspring groups, defined by maternal, postnatal and metformin exposure (C/C, Cm/C, C/HF, Cm/HF, HF/C, HFm/C, HF/HF and HFm/HF).

At 30 weeks, offspring were killed after an overnight fast via exsanguination and cervical dislocation after anaesthesia (using isoflurane). Plasma was separated, aliquoted and stored at -20°c for assessment of β-hydroxybutyrate levels. Livers were weighed and the left lobes were snap frozen in liquid nitrogen for later use.

7.2.2 Characterization of hepatic lipid composition

Gas chromatography was used to analyse hepatic lipid species in adult offspring liver, as described in detail in Chapter 3. Briefly, frozen left lobe liver samples (100mg) from adult offspring were homogenized in ice-cold NaCl solution and the homogenate vortexed with a 2:1 chloroform/methanol mix. Internal standards for TAGs, CEs, PCs and NEFAs were added and lipid fractions were separated by solid phase extraction on aminopropylsilica cartridges. CE and TAGs were eluted from the columns with chloroform; PC was eluted with 3:2 chloroform/methanol; and NEFA was eluted using 50:1:1 chloroform/methanol/glacial acetic acid. CEs were separated from TAGs by eluting with hexane. Fatty acid methyl esters (FAMEs) were formed by heating with methanol and H_2SO_4.

Samples were separated on a Hewlett-Packard 6890 gas chromatograph fitted with a BPX-70 column. FAMEs were detected and quantified using flame ionization detection. Retention times were compared against previously run authentic standards to identify FAME species in samples. Species mass was calculated and expressed per 100mg of liver tissue.

Table 22 | **Identified hepatic fatty acid species and groupings by class.** MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Common name	Lipid number
SFAs	
Myristic acid	14:0
Palmitic acid	16:0
Stearic acid	18:0
Arachidic acid	20:0
MUFAs	
Palmitoleic acid	16:1
Vaccenic acid	18:1n-7
Oleic acid	18:1n-9
Gondoic acid	20:1n-9
Erucic acid	22:1n-9
Nervonic acid	24:1n-9
n-3 PUFAs	
Alpha linolenic acid (ALA)	18:3n-3
Eicosapentaenoic acid (EPA)	20:5n-3
Docosapentaenoic acid n-3 (DPA)	22:5n-3
Docosahexaenoic acid (DHA)	22:6n-3
n-6 PUFAs	
Linoleic acid	18:2n-6
Gamma-linoleic acid	18:3n-6
Dihomo gamma-linoleic acid	20:3n-6
Arachidonic acid	20:4n-6
Docosadienoic acid	22:2n-6
Adrenic acid	22:4n-6
Docosapentaenoic acid n-6 (DPA)	22:5n-6

7.2.3 Hepatic gene expression

PCR was performed on isolated hepatic mRNA, as described in detail in Chapter 3. Briefly, total RNA was extracted from homogenized frozen liver tissue using Tri-reagent, separation using 1-bromo-chloropropane and precipitation with isopropanol. Sample purity and concentration was assessed by NanoDrop spectrophotometry. cDNA was synthesised using M-MLV reverse transcriptase according to the manufacturer's instructions.

Real-time qPCR was performed on genes of interest involved in hepatic lipid metabolism and DNL using TAQMAN probes primers and reagents from Eurogentec (*Srebp1*, *Ppara, Pparg*, *Lpl, Ywhaz, Sirt1*) and PrimerDesign (*Fas*, *Acc1*, *Acly, Cpt1*) on an ABI7500 real-time PCR system and reaction

conditions specific to the manufacturer's instructions. Gene expression was normalized to *Ywhaz* and then to the C/C group using the ΔΔCt method.

7.2.4 Quantification of hepatic mitochondrial copy number

Total cellular DNA (nuclear and mitochondrial) was extracted from 20mg of adult offspring liver samples as per the methods described in Chapter 3. Briefly, homogenized liver was processed using a Quick-gDNA Miniprep kit (Zymo Research) according to the manufacturer's instructions. PCR was performed on isolated DNA using primers specific for nuclear DNA (*Ywhaz*) and mitochondrial DNA (NADH dehydrogenase 4 (*Nd4*)). Relative copy number was defined as the relative expression of *Nd4* to *Ywhaz*.

7.2.5 Plasma β-hydroxybutyrate levels

Aliquots of offspring plasma were sent to the Core Biochemical Assay Laboratory, Cambridge, UK, for analysis. Levels were measured using the Stanbio Beta Hydroxybutyrate Liquicolour kit (Texas, USA), run according to the manufacturer's instructions on 10µl of plasma.

7.2.6 Data analysis

Data were analysed by mixed effects model as described in detail in Chapter 3. Briefly, maternal diet, postnatal diet and maternal metformin treatment were included as the main effects. Litter of origin was included as the random effects term, and litter size was included as an additional fixed effect to account for potential litter size effects. Maximal models were created using all main effects and interaction terms; interaction terms were removed if they were not significant. In addition to the primary model, secondary maximal models to calculate planned comparisons between non-metformin-treated groups and the respective metformin-treated group (e.g., C/C vs Cm/C offspring) were constructed. Predicted groups means and standard errors were used from these models to graph data. Data is reported as predicted effect sizes, SEM and P value for each significant factor in the text. Graphs show significant planned comparison tests using asterisks.

Tables accompany each figure and report and summarize significant fixed effects for each respective dependent variable

Before statistical modelling, all data were assessed for normality as described in Chapter 3. All PCR data were log-transformed for statistical analysis but graphed as non-logged data for clarity.

7.3 Results

7.3.1 Total hepatic lipid content in male and female offspring

Total assayed hepatic lipid (comprising the sum of TAGs, NEFAs, PCs and CEs) in female offspring was assessed using gas chromatography (Figure 29, Table 23). Levels were only increased by postnatal HF diet (FE 143.2 ± 34.8 mg/g tissue, P < 0.0001). When broken down by lipid fraction, TAGs were increased only by postnatal HF diet (FE 43.7 ± 17.6mg/g tissue, P = 0.02). CEs were increased by postnatal HF diet (FE 104.5 ± 19.8 mg/g tissue, P < 0.0001), but also by maternal diet (FE 63.9 ± 26.2 mg/g tissue, P = 0.027). In planned comparison tests, HFm/C offspring had twofold lower hepatic CE levels than HF/C offspring (FE -100.1 ± 44.9 mg/g tissue, P = 0.035). There were no differences in the levels of PCs or NEFAs between offspring groups.

Dependent variable	Significant effects	Fixed effect est.	P
Female total hepatic lipid	Postnatal HF diet	143.2 ± 34.8 mg/g	< 0.0001
Female hepatic TAG	Postnatal HF diet	43.7 ± 17.6 mg/g	= 0.02
Female hepatic CE	Postnatal HF diet Maternal HF diet	104.5 ± 19.8 mg/g 63.9 ± 26.2 mg/g	< 0.0001 = 0.027
Female hepatic PC	NONE	NA	NA
Female hepatic NEFA	NONE	NA	NA

Table 23 | Statistical results from Figure 29. CE, cholesterol ester; HF, high-fat; NA, not applicable; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; TAG, triglyceride.

Dependent variable	Significant effects	Fixed effect est.	P
Female total hepatic lipid	Postnatal HF diet	143.2 ± 34.8 mg/g	< 0.0001
Female hepatic TAG	Postnatal HF diet	43.7 ± 17.6 mg/g	= 0.02
Female hepatic CE	Postnatal HF diet Maternal HF diet	104.5 ± 19.8 mg/g 63.9 ± 26.2 mg/g	< 0.0001 = 0.027
Female hepatic PC	NONE	NA	NA
Female hepatic NEFA	NONE	NA	NA

Table 24 | Statistical results from Figure 30. CE, cholesterol ester; HF, high-fat; NA, not applicable; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; TAG, triglyceride.

In males, total hepatic lipid (Figure 30, Table 24) was markedly increased by postnatal HF diet (FE 156.8 ± 29.7 mg/g tissue, P < 0.0001) and also by metformin treatment (FE 126.2 ± 43.9 mg/g tissue, P = 0.014). Notably, total hepatic lipid in HFm/HF males was greatly increased relative to HF/HF males (FE 209 ± 67.6 mg/g tissue, P = 0.006). TAG comprised the largest fraction of total hepatic lipids in male offspring in all eight groups. Postnatal HF diet was the strongest contributor to hepatic TAG accumulation (FE 112.7 ± 22.6 mg/g tissue, P < 0.0001), yet the presence of metformin treatment in mothers also increased offspring TAG (FE 80.9 ± 32.5 mg/g tissue, P = 0.032). In planned comparison tests the increased TAG in HFm/HF vs HF/HF offspring was significant (FE 162.2 ± 48.4 mg/g tissue, P = 0.004). CE levels were increased by maternal HF diet (FE 39.9 ± 16.4 mg/g tissue, P = 0.028), maternal metformin treatment (FE 43.1 ± 17.2 mg/g

tissue, P = 0.025) and by postnatal HF diet (45.9 ± 11.8 mg/g tissue, P < 0.001). As in females, PCs were unaltered between offspring groups in males, yet NEFAs were significantly increased by maternal HF diet (FE 1.1 ± 0.4 mg/g tissue, P = 0.01). In addition, NEFAs were decreased by a combination of maternal and postnatal HF diets (FE – 0.8 ± 0.4 mg/g tissue, P = 0.035), and by maternal metformin treatment in obese dams only (FE -1.1 ± 0.5 mg/g tissue, P = 0.037).

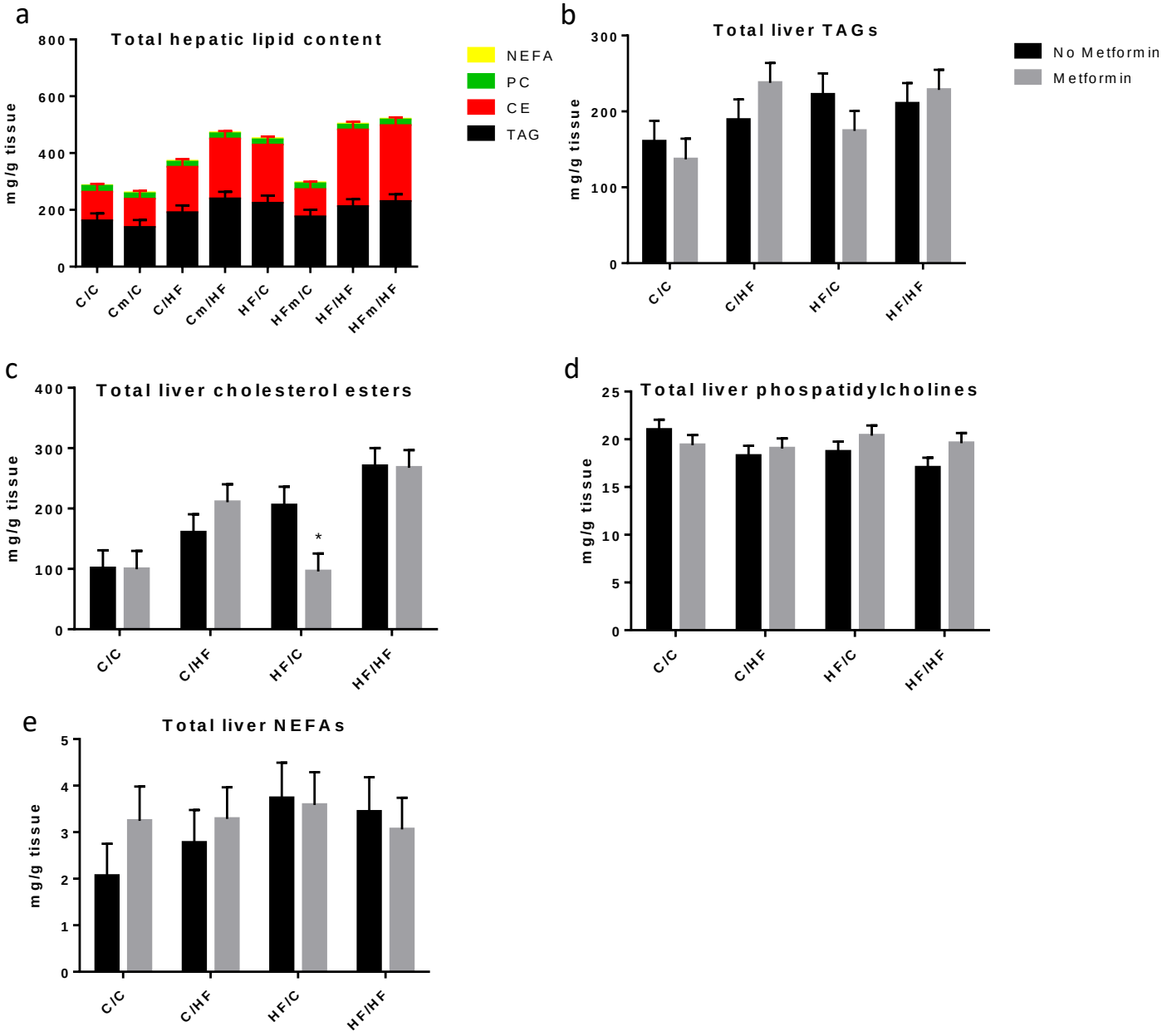

Figure 29 | **Hepatic lipids by fraction in adult female offspring.** Significant main effects and interactions are discussed in the main text. *, P < 0.05 vs respective non-treated diet group in planned comparison tests. All data presented as predicted means ± SEM from maximal models. C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). C, chow; CE, cholesterol ester; HF, high-fat; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; TAG, triglyceride.

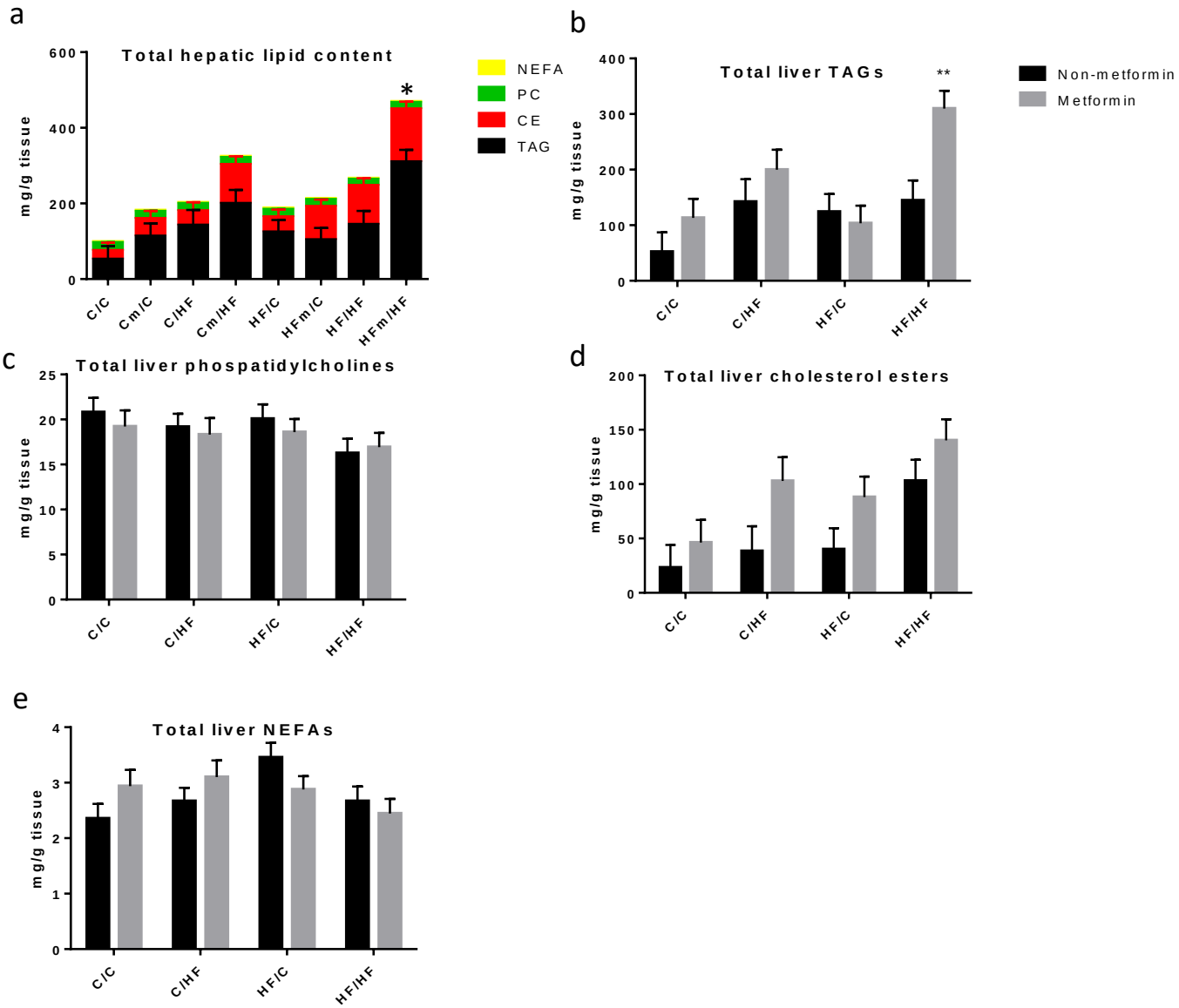

Figure 30 | **Hepatic lipids by fraction in adult male offspring.** Significant main effects and interactions are discussed in the main text.*, P < 0.05; **, P < 0.01; vs respective non-treated diet group in planned comparison tests. All data presented as predicted means ± SEM from maximal models. C/C, n = 5 (from 3 litters); Cm/C, n = 5 (from 5 litters); C/HF, n = 5 (from 2 litters); Cm/HF, n = 5 (from 4 litters); HF/C, n = 5 (from 4 litters); HFm/C, n = 5 (from 3 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 4 litters). C, chow; CE, cholesterol ester; HF, high-fat; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; TAG, triglyceride.

7.3.2 Hepatic lipid content by class

Next, individual fatty acids in all liver fractions were grouped by class into SFAs, n-3 PUFAs, n-6 PUFAs and MUFAs. Total hepatic SFA levels in females (Figure 31, Table 25) were significantly increased only by postnatal HF diet (FE 39.8 ± 8.5 mg/g tissue, P < 0.0001). MUFAs were increased independently by both maternal HF diet (FE 57.5 ± 24.5 mg/g tissue, P = 0.032) and postnatal HF diet (83.7 ± 19.1 mg/g tissue, P < 0.0001). By contrast, total levels of n-3 and n-6 PUFAs were

unchanged across groups. However, the ratio of n-3:n-6 PUFAs in females was decreased by postnatal HF diet (FE -0.027 ± 0.01, P = 0.009).

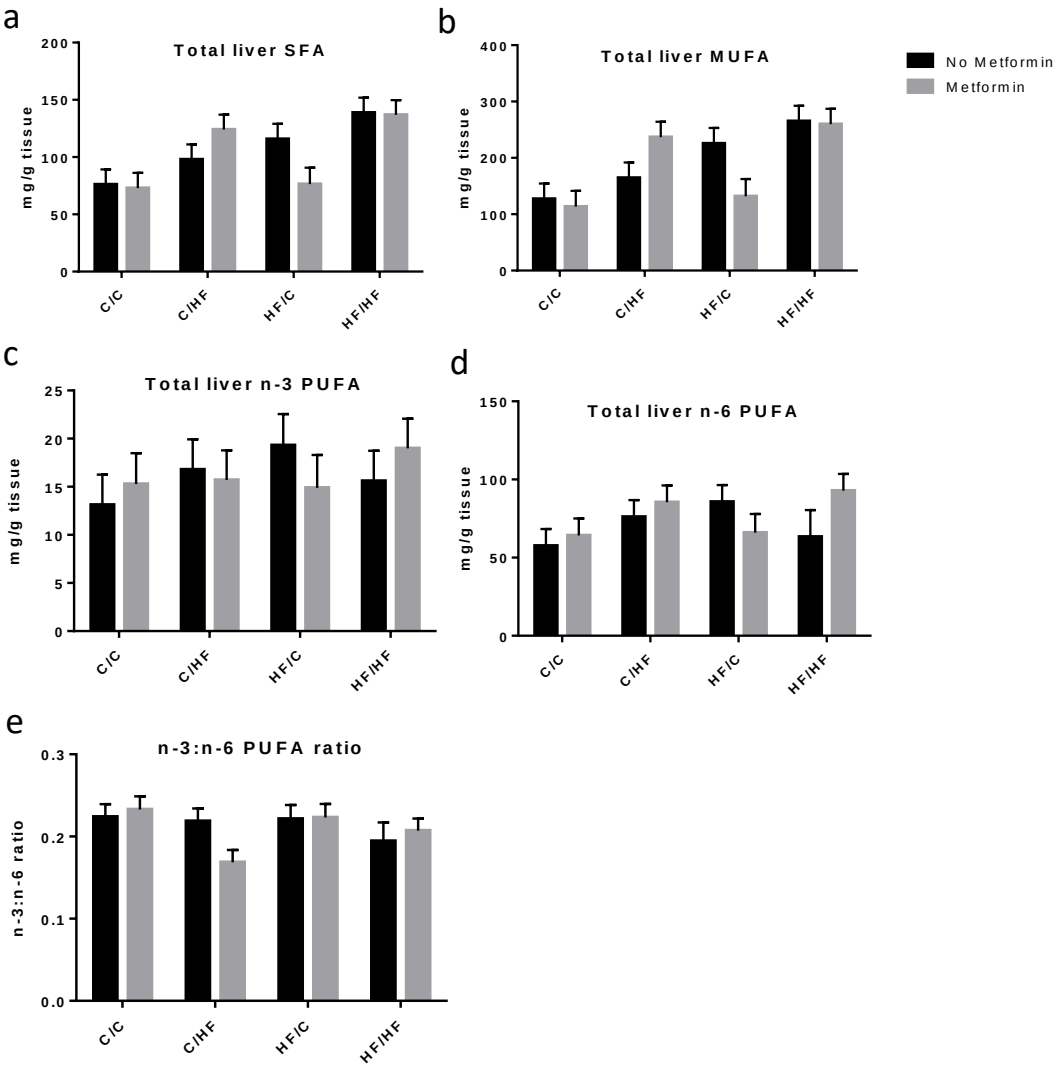

Figure 31 | **Hepatic lipids by class in adult female offspring.** All data presented as predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). C, chow; HF, high-fat; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Female hepatic SFA	Postnatal HF diet	39.8 ± 8.5 mg/g	< 0.0001
Female hepatic MUFA	Maternal HF diet Postnatal HF diet	57.5 ± 24.5 mg/g 83.7 ± 19.1 mg/g	= 0.032 < 0.0001
Female hepatic n-3 PUFAs	NONE	NA	NA
Female hepatic n-6 PUFAs	NONE	NA	NA
Female hepatic n-3:n-6 PUFA	Postnatal HF diet	-0.027 ± 0.01	= 0.009

Table 25 | Statistical results from Figure 31. HF, high-fat; MUFA, monounsaturated fatty acid; NA, not applicable; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Levels of lipid classes in female offspring were assessed in TAG, CE, PC and NEFA fractions and are shown in

Table 26. Pairwise (non-metformin vs respective metformin treated group) post-hoc testing was conducted to explore whether metformin might have altered individual lipid classes dependent on the lipid fraction. These data show that HFm/HF offspring had significantly more n-3 PUFAs in the PC and NEFA fractions, whereas levels in the TAG and CE fractions were not significantly different.

Table 26 | **Female adult offspring hepatic lipid fractions separated by constituent classes.** Values are mg/g liver tissue except for n-3:n-6 ratios. Data are predicted means ± SEM derived from maximal models. *, P < 0.05 vs respective untreated diet group in planned comparison tests. C, chow; CE, cholesterol ester; HF, high-fat; m, metformin; MUFA, monounsaturated fatty acids; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAG, triglyceride.

	C/C (n = 5)	**Cm/C** (n = 5)	**C/HF** (n = 5)	**Cm/HF** (n = 5)	**HF/C** (n = 5)	**HFm/C** (n = 5)	**HF/HF** (n = 5)	**HFm/HF** (n = 5)
TAG								
SFA	37.89 ± 5.79	36.98 ± 5.85	44.06 ± 5.80	56.73 ± 5.79	51.98 ± 5.83	39.55 ± 5.77	56.48 ± 5.80	54.78 ± 5.77
MUFA	72.06 ± 12.17	61.72 ± 12.31	81.43 ± 12.19	**116.76 ± 12.03***	106.53 ±12.37	77.84 ± 11.99	107.02 ± 12.18	110.27 ± 12.01
n-6 PUFA	38.11 ± 6.85	42.84 ± 6.92	45.17 ± 6.86	50.42 ± 6.86	49.25 ± 6.87	43.34 ± 6.84	44.94 ± 6.85	47.68 ± 6.85
n-3 PUFA	8.95 ± 2.56	11.03 ± 2.59	11.08 ± 2.57	10.24 ± 2.53	13.75 ± 2.61	9.39 ± 2.52	9.72 ± 2.56	11.66 ± 2.53
n-3:n-6 ratio	0.23 ± 0.02	0.24 ± 0.02	0.24 ± 0.02	0.17 ± 0.02	0.28 ± 0.03	0.21 ± 0.02	0.21 ± 0.02	0.25 ± 0.02
CE								
SFA	28.75 ± 8.29	26.99 ± 8.40	44.58 ± 8.31	58.29 ± 8.14	55.65 ± 8.49	**26.95 ± 8.11***	73.93 ± 8.30	72.94 ±8.13
MUFA	51.70 ± 16.53	48.21 ± 16.70	80.71 ± 16.55	116.77 ± 16.45	114.91 ± 16.68	**49.68 ± 16.40***	154.71 ± 16.53	146.51 ± 16.42
n-6 PUFA	17.97 ± 5.19	17.96 ± 5.31	29.68 ± 5.22	31.64 ± 5.02	32.50 ± 5.46	17.08 ± 5.00	37.33 ± 5.25	42.17 ± 5.01
n-3 PUFA	1.17 ± 0.66	1.28 ± 0.67	2.68 ± 0.66	2.30 ± 0.64	2.97 ± 0.69	1.25 ± 0.64	2.84 ± 0.66	3.60 ± 0.63
n-3:n-6 ratio	0.034 ± 0.011	0.027 ± 0.011	0.062 ± 0.011	0.042 ± 0.010	0.057 ± 0.011	0.028 ± 0.010	0.059 ± 0.011	0.076 ± 0.010
PC								
SFA	8.84 ± 0.42	8.02 ± 0.42	7.90 ± 0.42	7.85 ± 0.42	7.91 ± 0.42	8.61 ± 0.42	7.10 ± 0.42	8.11 ± 0.42
MUFA	2.63 ± 0.18	2.46 ± 0.18	1.95 ± 0.18	2.15 ± 0.17	2.59 ± 0.18	2.47 ± 0.17	1.86 ± 0.18	2.17 ± 0.17
n-6 PUFA	2.77 ± 0.17	2.72 ± 0.17	2.38 ± 0.17	2.51 ± 0.17	2.45 ± 0.17	2.64 ± 0.17	1.96 ± 0.17	2.21 ± 0.17
n-3 PUFA	2.86 ± 0.19	2.67 ± 0.19	2.77 ± 0.19	2.87 ± 0.18	2.33 ± 0.20	2.80 ± 0.18	2.71 ± 0.19	**3.35 ± 0.18***
n-3:n-6 ratio	1.05 ± 0.07	1.01 ± 0.07	1.16 ± 0.07	1.16 ± 0.07	0.99 ± 0.07	1.06 ± 0.07	1.41 ± 0.07	1.52 ± 0.07
NEFA								
SFA	0.81 ± 0.21	1.10 ± 0.22	1.04 ± 0.21	1.15 ± 0.20	1.25 ± 0.23	1.18 ± 0.22	1.18 ± 0.22	1.01 ± 0.20
MUFA	0.58 ± 0.32	0.89 ± 0.34	0.76 ± 0.33	1.23 ± 0.32	1.36 ± 0.36	1.22 ± 0.33	1.33 ± 0.35	1.03 ± 0.32
n-6 PUFA	0.27 ± 0.06	0.48 ± 0.06	0.34 ± 0.06	0.43 ± 0.06	0.33 ± 0.07	0.54 ± 0.07	0.27 ± 0.09	0.41 ± 0.06
n-3 PUFA	0.21 ± 0.03	0.29 ± 0.03	0.30 ± 0.03	0.26 ± 0.03	0.25 ± 0.03	0.32 ± 0.03	0.25 ± 0.03	**0.36 ± 0.03***
n-3:n-6 ratio	0.81 ± 0.11	0.64 ± 0.11	0.93 ± 0.11	0.74 ± 0.11	0.77 ± 0.11	0.66 ± 0.12	0.64 ± 0.17	0.89 ± 0.11

In males (Figure 32, Table 27), total hepatic SFA was increased by the independent effects of maternal metformin treatment (FE 34.4 ± 11.0 mg/g tissue, P = 0.009) and postnatal HF diet (FE 40.4 ± 7.9 mg/g tissue, P < 0.0001). Planned comparison tests showed that the increased SFA resulting from metformin treatment was largely the result of SFA accumulation in HFm/HF vs HF/HF offspring (FE 57.4 ± 17.4 mg/g tissue, P = 0.004). Similarly, MUFAs were also increased by metformin treatment (FE 75.1 ± 23.9 mg/g tissue, P = 0.008) and by postnatal HF diet (FE 90.1 ± 16.3 mg/g tissue, P < 0.0001). In planned comparison tests, increased MUFA accumulation was

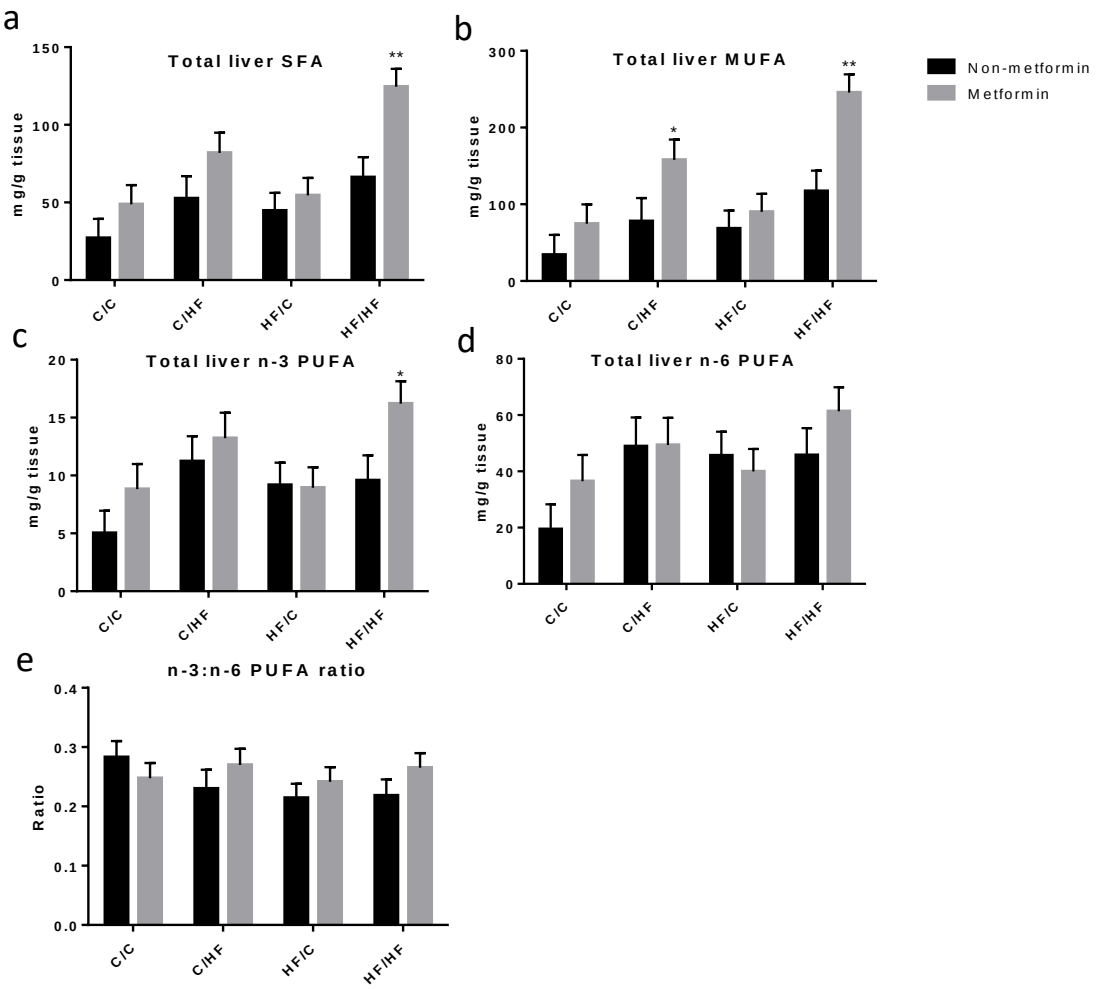

Figure 32 | **Hepatic lipids by class in adult male offspring.** Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; vs respective non-metformin group in planned comparison tests. C< chow; HF, high-fat; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids. All data presented as predicted means ± SEM from maximal models. C/C, n = 5 (from 3 litters); Cm/C, n = 5 (from 5 litters); C/HF, n = 5 (from 2 litters); Cm/HF, n = 5 (from 4 litters); HF/C, n = 5 (from 4 litters); HFm/C, n = 5 (from 3 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 4 litters).

seen in HFm/HF vs HF/HF offspring (FE 126.8 ± 36.8 mg/g tissue, P = 0.002), as in SFAs, but also in Cm/HF vs C/HF offspring (FE 108.0 ± 48.1 mg/g tissue, P = 0.036). Whereas n-3 PUFA levels were similar in all female offspring groups, they were significantly increased in males by postnatal HF diet (FE 4.8 ± 1.4 mg/g tissue, P = 0.002). Interestingly, planned comparison tests revealed

HFm/HF offspring had markedly higher n-3 PUFA levels than HF/HF offspring (FE 6.4 ± 2.9 mg/g tissue, P = 0.042). Postnatal HF diet also increased n-6 PUFA levels (FE 16.7 ± 5.9 mg/g tissue, P = 0.009). There was no alteration to the n-3:n-6 ratio in any of the groups.

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Male hepatic SFA	Maternal HF diet Postnatal HF diet	34.4 ± 11.0 mg/g 40.4 ± 7.9 mg/g	= 0.009 < 0.0001
Male hepatic MUFA	Postnatal HF diet Metformin	 90.1 ± 16.3 mg/g 75.1 ± 23.9 mg/g	< 0.001 = 0.008
Male hepatic n-3 PUFAs	Postnatal HF diet	4.8 ± 1.4 mg/g	= 0.002
Male hepatic n-6 PUFAs	Postnatal HF diet	16.7 ± 5.9 mg/g	= 0.009
Male hepatic n-3:n-6 PUFA	NONE	NA	NA

Table 27 | Statistical results from Figure 32. HF, high-fat; MUFA, monounsaturated fatty acid; NA, not applicable; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 28 | **Male adult offspring hepatic lipid fractions separated by constituent classes.** Values are mg/g liver tissue, except n-3:n-6 ratio. Data are predicted means ± SEM. *, P < 0.05 vs respective non-treated diet groups in planned comparison tests. C, chow; CE, cholesterol ester; HF, high-fat; MUFA, monounsaturated fatty acids; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAG, triglyceride.

	C/C (n = 5)	**Cm/C** (n = 5)	**C/HF** (n = 5)	**Cm/HF** (n = 5)	**HF/C** (n = 5)	**HFm/C** (n = 5)	**HF/HF** (n = 5)	**HFm/HF** (n = 5)
TAG								
SFA	12.41 ± 8.73	28.48 ± 8.49	33.27 ± 10.19	48.37 ± 8.94	27.98 ± 7.92	24.83 ± 7.88	35.17 ± 8.98	**79.56 ± 7.98***
MUFA	23.06 ± 17.90	52.65 ± 17.24	56.84 ± 20.92	101.77 ± 18.18	51.60 ± 16.12	47.03 ± 16.15	67.00 ± 18.26	**166.36 ± 16.29***
n-6 PUFA	12.93 ± 7.42	24.41 ± 7.67	38.79 ± 8.56	34.89 ± 7.98	34.43 ± 7.04	23.67 ± 6.71	31.72 ± 7.96	44.90 ± 7.04
n-3 PUFA	2.06 ± 1.93	5.24 ± 2.02	7.78 ± 2.22	9.34 ± 2.09	6.17 ± 1.85	5.13 ± 1.75	6.47 ± 2.08	12.41 ± 1.85
n-3:n-6 ratio	0.158 ± 0.020	0.220 ± 0.021	0.187 ± 0.24	0.268 ± 0.022	0.165 ± 0.019	0.217 ± 0.018	0.203 ± 0.022	**0.269 ± 0.019***
CE								
SFA	6.65 ± 5.43	11.86 ± 5.45	8.86 ± 5.96	25.01 ± 5.71	8.25 ± 5.03	21.72 ± 4.91	26.51 ± 5.03	37.25 ± 5.06
MUFA	9.19 ± 11.02	18.29 ± 10.83	16.39 ± 12.24	**51.96 ± 11.39***	13.06 ± 10.03	39.87 ± 9.96	53.07 ± 10.03	75.95 ± 10.15
n-6 PUFA	2.95 ± 2.95	8.45 ± 2.96	5.62 ± 3.23	11.97 ± 3.10	7.24 ± 2.73	12.94 ± 2.66	12.08 ± 2.73	14.18 ± 2.75
n-3 PUFA	0.09 ± 0.29	0.79 ± 0.30	0.36 ± 0.31	1.09 ± 0.31	0.52 ± 0.28	1.26 ± 0.26	1.23 ± 0.28	1.52 ± 0.28
n-3:n-6 ratio	0.006 ± 0.019	0.034 ± 0.018	0.006 ± 0.021	0.031 ± 0.019	0.007 ± 0.017	0.056 ± 0.017	0.073 ± 0.019	0.033 ± 0.017
PC								
SFA	8.27 ± 0.66	7.56 ± 0.74	7.87 ± 0.60	7.14 ± 0.75	8.01 ± 0.66	7.30 ± 0.60	6.39 ± 0.66	6.77 ± 0.66
MUFA	2.61 ± 0.27	2.68 ± 0.27	2.17 ± 0.29	**2.53 ± 0.29***	2.51 ± 0.25	2.69 ± 0.25	2.34 ± 0.25	2.43 ± 0.25
n-6 PUFA	3.65 ± 0.33	3.11 ± 0.34	3.33 ± 0.35	2.36 ± 0.35	3.58 ± 0.31	2.99 ± 0.30	2.42 ± 0.31	2.02 ± 0.31
n-3 PUFA	2.81 ± 0.25	2.51 ± 0.28	2.69 ± 0.23	2.59 ± 0.28	2.48 ± 0.25	2.52 ± 0.24	2.08 ± 0.25	2.20 ± 0.25
n-3:n-6 ratio	0.823 ± 0.083	0.806 ± 0.078	0.861 ± 0.092	1.072 ± 0.08	0.654 ± 0.072	**0.887 ± 0.075***	0.915 ± 0.072	1.11 ± 0.08
NEFA								
SFA	0.80 ± 0.10	0.90 ± 0.11	1.02 ± 0.09	1.03 ± 0.11	1.22 ± 0.10	**0.93 ± 0.09***	0.97 ± 0.10	0.93 ± 0.10
MUFA	0.57 ± 0.09	0.90 ± 0.10	0.67 ± 0.08	**1.12 ± 0.11***	0.95 ± 0.09	0.89 ± 0.08	0.99 ± 0.09	0.88 ± 0.09
n-6 PUFA	0.43 ± 0.6	0.56 ± 0.07	0.45 ± 0.06	0.40 ± 0.07	0.66 ± 0.06	0.55 ± 0.06	0.32 ± 0.06	0.22 ± 0.06
n-3 PUFA	0.28 ± 0.03	0.29 ± 0.04	0.27 ± 0.03	0.28 ± 0.04	0.30 ± 0.03	0.26 ± 0.03	0.19 ± 0.03	0.21 ± 0.03
n-3:n-6 ratio	0.656 ± 0.094	0.56 ± 0.10	0.59 ± 0.10	0.73 ± 0.10	0.45 ± 0.09	0.54 ± 0.09	0.62 ± 0.09	**0.96 ± 0.09***

Levels of fatty acid classes in each fraction for male offspring are shown in Table 28. These data show that the relative proportions of each fatty acid class can differ by fraction. HFm/HF offspring show significantly increased SFA and MUFA levels only in the TAG fraction. However, Cm/HF

offspring, who showed increased levels of MUFA, have significantly raised levels in all fractions except TAGs (in which there is a trend towards an increase).

DNL, SCD and n-3 and n-6 product:precursor ratios were calculated for both male and female offspring (Figure 33, Table 29). DNL ratio in females was significantly increased by the independent effects of maternal HF diet (FE 0.28 ± 0.10, P = 0.007) and by postnatal HF diet (FE 0.18 ± 0.07, P = 0.009). Moreover, maternal metformin treatment in HF dams reduced the DNL in offspring (FE -0.29 ± 0.13, P = 0.04). In planned comparison tests, Cm/HF offspring had a significantly higher DNL ratio than C/HF offspring (FE 0.34 ± 0.14, P = 0.022). The SCD ratio, reflecting SCD activity, was also increased by maternal (FE 3.2 ± 1.1, P = 0.011) and postnatal (FE 2.1 ± 1.0, P = 0.043) HF diets. There was no interaction between maternal diet and metformin treatment. The increased SCD ratio between Cm/HF and C/HF offspring was also significant (FE 4.9 ± 2.1, P = 0.025). The product:precursor ratio for n-3 PUFAs (the ratio of EPA to ALA, indicating activity of Δ6 and Δ5 desaturases) was increased by maternal metformin treatment in offspring of HF-fed dams (FE 0.20 ± 0.09, P = 0.033). The product:precursor ratio for n-6 PUFAs (the ratio of arachidonic acid to linoleic acid) was decreased by postnatal HF diet (FE –0.036 ± 0.007, P < 0.0001).

Dependent variable	**Significant effects**	**Fixed effect est.**	**P**
Female DNL index	Maternal HF diet	0.28 ± 0.10	= 0.007
	Postnatal HF diet	0.18 ± 0.07	= 0.009
	Metformin * Maternal HF diet	-0.29 ± 0.13	= 0.040
Female SCD index	Maternal HF diet	3.2 ± 1.1	= 0.011
	Postnatal HF diet	2.1 ± 1.0	= 0.043
Female n-3 product:precursor	Metformin * Maternal HF diet	0.20 ± 0.09	= 0.033
Female n-6 product:precursor	Postnatal HF diet	-0.036 ± 0.007	< 0.0001
Male DNL index	Metformin	0.47 ± 0.13	= 0.004
	Maternal HF diet * Postnatal HF diet	0.68 ± 0.19	< 0.001
Male SCD index	Maternal HF diet	5.6 ± 2.5	= 0.042
	Postnatal HF diet	8.0 ± 1.9	< 0.0001
	Metformin	8.6 ± 2.6	= 0.006
Male n-3 product:precursor	Maternal HF diet	-0.24 ± 0.08	= 0.012
	Postnatal HF diet	-0.21 ± 0.06	= 0.002
Male n-6 product:precursor	NONE	NA	NA

Table 29 | Statistical results for Figure 33. DNL, *de novo* lipogenesis; HF, high-fat; NA, not applicable; SCD, steroyl-CoA desaturase.

In male offspring, DNL ratio was increased independently by maternal metformin treatment (FE 0.47 ± 0.13, P = 0.004). There was a significant interaction between maternal and postnatal HF diets, which increased DNL ratio in offspring exposed to both (FE 0.68 ± 0.19, P < 0.001). SCD ratio was increased independently by maternal HF diet (FE 5.6 ± 2.5, P = 0.042), by postnatal HF diet (FE 8.0 ± 1.9, P < 0.0001) and by maternal metformin treatment (FE 8.6 ± 2.6, P = 0.006). The increased SCD ratio observed in HFm/HF offspring compared with HF/HF offspring was significant in planned comparison tests (FE 12.4 ± 4.4, P = 0.012). Product:precursor ratio for n-3 PUFAs

reduced by maternal HF diet (FE -0.24 ± 0.08, P = 0.012) and by postnatal HF diet (FE -0.21 ± 0.06, P = 0.002). However, combined maternal and postnatal HF diet did not combine to exacerbate the reduction in n-3 product:precursor ratio. Interestingly, the reduced ratio in Cm/C vs C/C offspring was significant in planned comparison tests (FE -0.35 ± 0.14, P < 0.05). The product:precursor ratio for n-6 PUFAs was not significantly altered between groups.

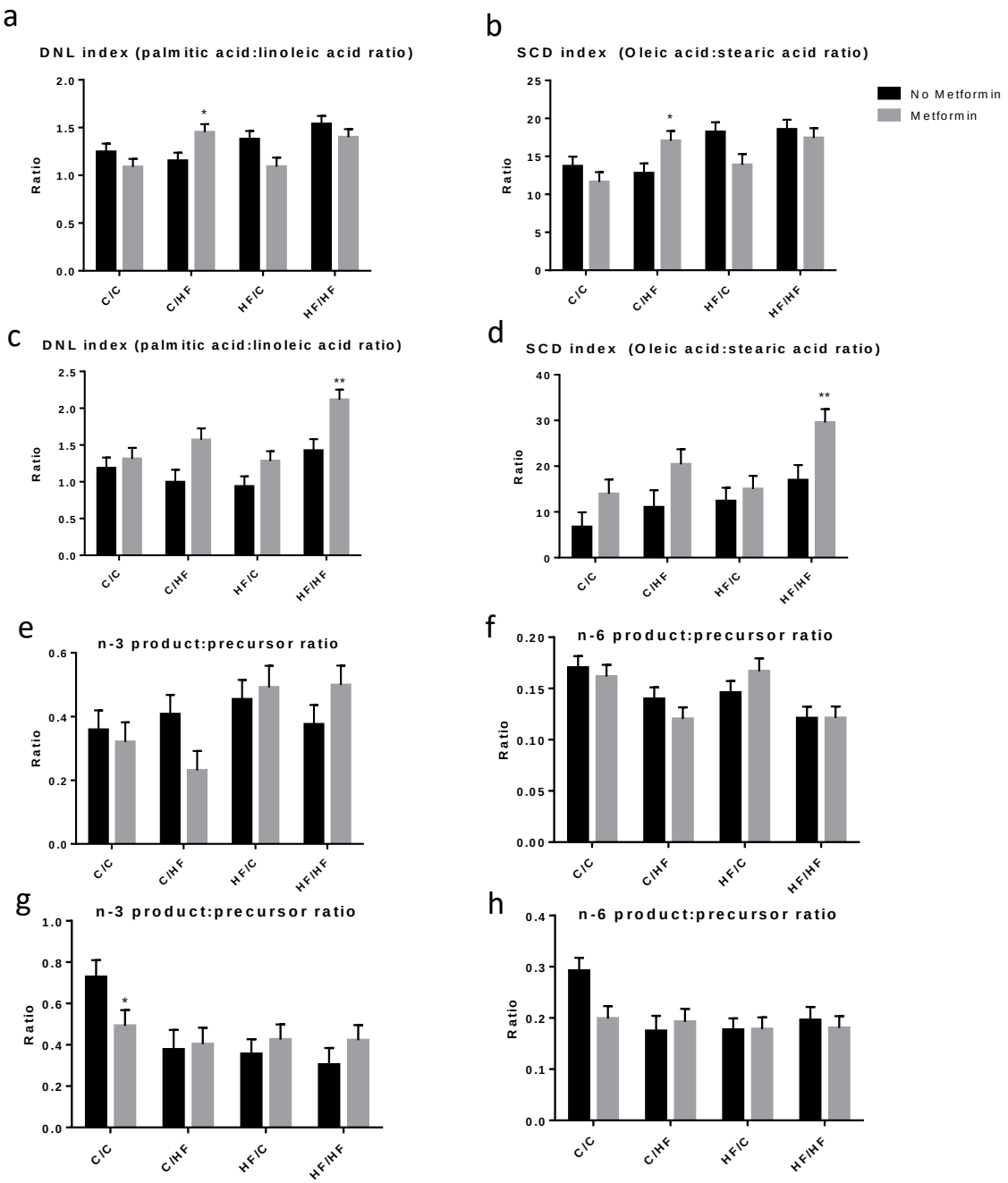

Figure 33 | **Hepatic DNL, desaturase and n-3 and n-6 production indices. a**. Hepatic DNL index in adult female offspring. **b**. Hepatic Δ9 desaturase index in adult female offspring. **c**. Hepatic DNL index in adult male offspring. **d**. Hepatic Δ9 desaturase index in adult male offspring**. e**. n-3 product:precursor ratio in adult female offspring. **f**. n-6 product:precursor ratio in adult female offspring. **g**. n-3 product:precursor ratio in adult male offspring. **h**. n-6 product:precursor ratio in adult male offspring. Data are predicted means ± SEM from maximal models. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; vs respective non-metformin group in planned comparison tests. Females: C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 5 (from 5 litters); C/HF, n = 5 (from 2 litters); Cm/HF, n = 5 (from 4 litters); HF/C, n = 5 (from 4 litters); HFm/C, n = 5 (from 3 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 4 litters). C, chow; DNL, *de novo* lipogenesis;HF, high-fat; SCD, stearoyl-CoA desaturase.

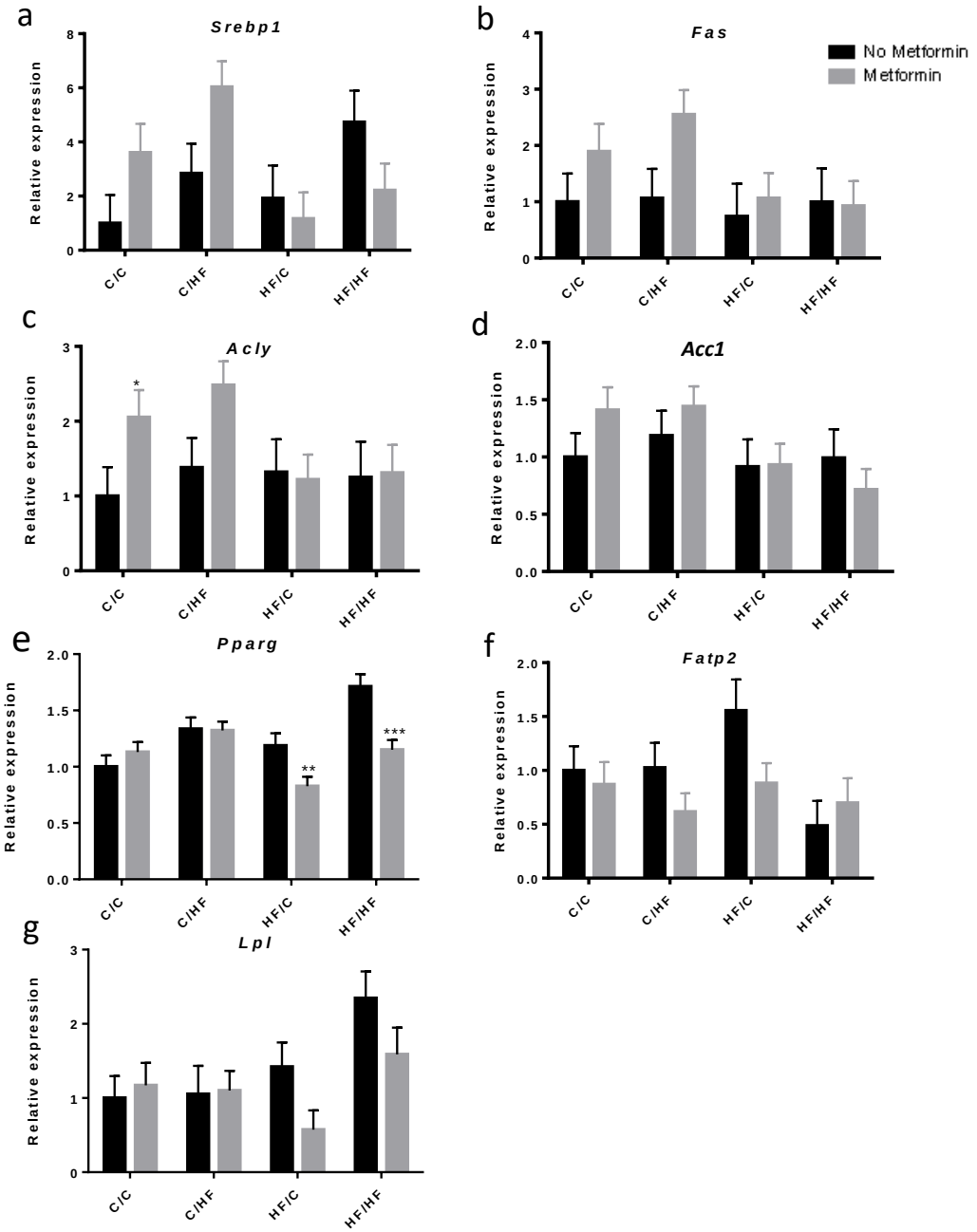

Figure 34 | **Expression of genes related to lipid homeostasis in adult female offspring.** Gene expression data are non-logged predicted means ± SEM. Statistics were performed on logged data. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; ***, P < 0.001 vs respective non-metformin group in planned comparison tests. C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters). C, chow; HF, high-fat.

Because DNL index was significantly altered in female adult offspring liver, hepatic expression of key lipogenic genes was assessed by qPCR (Figure 34, Table 30). Expression of *Srebp1*, encoding a master transcription factor regulating prolipogenic genes, SREBP1c, was increased by postnatal HF diet (logFE 0.35 ± 0.06, P < 0.0001). Despite the increased expression resulting from maternal diet not being significant, maternal metformin treatment did reduce *Srebp1* expression in offspring of

HF dams (logFE -0.55 ± 0.19, P = 0.013), largely as a result of reduced expression in HFm/HF vs HF/HF animals. The increased expression in offspring of Cm dams did not reach significance in planned comparison testing. Next, expression of lipogenic downstream targets of SREBP1c were assessed. *Acly* and *Fas* expression were not altered by maternal or postnatal diets, or by metformin treatment, despite trends towards increased expression of offspring of Cm dams. Expression of *Acc1* was decreased by maternal diet.

Pparg encodes the peroxisome-proliferator activated receptor (PPAR) family member PPARγ. Although normally expressed in adipose tissue, upregulation of this gene is seen in animal models of NAFLD. Expression was increased by maternal HF diet (logFE 0.092 ± 0.042, P = 0.042) and robustly by postnatal HF diet (logFE 0.124 ± 0.025, P < 0.0001). There was a significant effect of metformin in HF dams that markedly reduced *Pparg* expression (logFE –0.189 ± 0.052, P = 0.004). Expression of *Sirt1*, which was increased by metformin in fetal livers (Chapter 4), was upregulated only in Cm/C vs C/C offspring, and not by maternal HF diet, postnatal HF diet or broad metformin effects.

Dependent variable	Significant effects	Fixed effect est.	P
Srebp1	Postnatal HF diet Metformin * Maternal HF diet	0.35 ± 0.06 log AU -0.55 ± 0.19 log AU	< 0.0001 = 0.013
Acly	NONE	NA	NA
Fas	NONE	NA	NA
Acc1	Maternal HF diet	-0.15 ± 0.05 log AU	= 0.012
Pparg	Maternal HF diet Postnatal HF diet Metformin * Maternal HF diet	0.092 ± 0.042 log AU 0.124 ± 0.025 log AU -0.189 ± 0.052 log AU	= 0.042 < 0.0001 = 0.004
Sirt1	NONE	NA	NA
Fatp2	Maternal HF diet * Postnatal HF diet	-0.50 ± 0.21 log AU	= 0.024
Lpl	Metformin Maternal HF diet * Postnatal HF diet	-0.25 ± 0.12 log AU 0.45 ± 0.18 log AU	= 0.037 = 0.022

Table 30 | Statistical results for Figure 34. AU, arbitrary units; HF, high-fat; NA, not applicable.

Hepatocyte fatty acid transport dysregulation is implicated in NAFLD. Levels of fatty acid transport protein 2 (*Fatp2*), which is responsible for a large proportion of hepatocyte fatty acid uptake (40% in one study[429]), were decreased by the combined effect of maternal and postnatal HF diets (logFE 0.50 ± 0.21, P = 0.024) but not by the individual, independent diets, and there were no effects of metformin. Expression of lipoprotein lipase (*Lpl*), which is usually only expressed at high levels in fetal liver in mice but is upregulated in patients with NAFLD, was increased as a result of combined maternal and postnatal HF diet (logFE 0.45 ± 0.18, P = 0.022), but not by independent maternal or postnatal HF diet exposure. Metformin treatment reduced *Lpl* expression (logFE -0.25 ± 0.12, P = 0.037), a finding shown in planned comparison tests to be largely due to reduced expression in HFm/C vs HF/HF offspring (logFE -0.55 ± 0.18, P = 0.006).

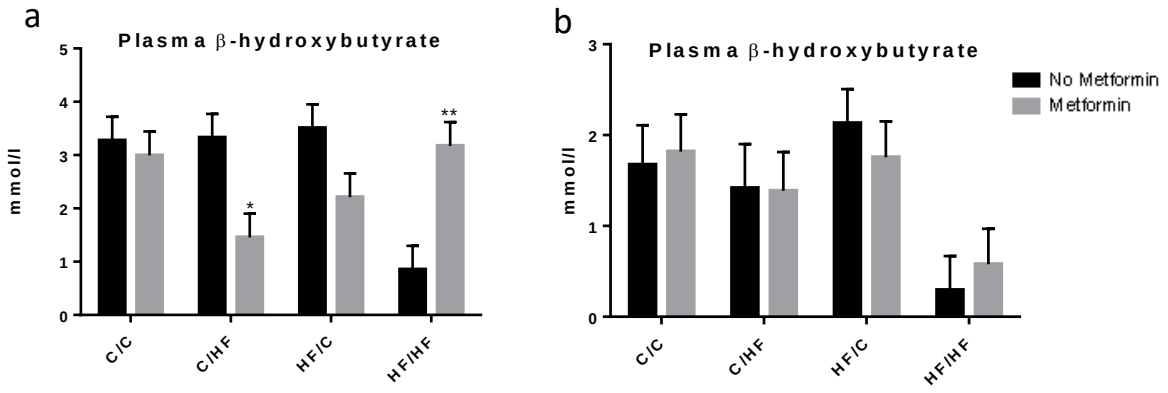

Figure 35 | **Offspring plasma β-hydroxybutyrate levels. a**. Plasma levels of β-hydroxybutyrate in adult female offspring. **b**. Plasma levels of β-hydroxybutyrate in adult male offspring. Plasma β-hydroxybutyrate levels are predicted means ± SEM. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01; vs respective non-metformin group in planned comparison tests. Females: C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 5 (from 5 litters); C/HF, n = 5 (from 2 litters); Cm/HF, n = 5 (from 4 litters); HF/C, n = 5 (from 4 litters); HFm/C, n = 5 (from 3 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 4 litters). C, chow; HF, high fat.

During fasting, β-oxidation in the liver releases ketone bodies to the circulation, which serve as an energy source in extrahepatic tissues. Because β-oxidation is a key route of fatty acid removal from the liver, plasma levels of β-hydroxybutyrate were assessed (Figure 35, Table 31). In female offspring, although maternal and postnatal HF diets or metformin treatment did not independently affect plasma β-hydroxybutyrate concentration, the combined effect of maternal and postnatal HF diets did dramatically reduce levels (FE -2.70 ± 0.89 mmol/l, P = 0.006). In planned comparison tests, metformin significantly rescued this reduction in HFm/HF vs HF/HF offspring (FE 2.31 ± 0.66 mmol/l, P = 0.002). Metformin treatment reduced β-hydroxybutyrate levels in Cm/HF vs C/HF offspring (FE -1.89 ± 0.73 mmol/l, P = 0.018).

In male offspring (Figure 35b, Table 31), β-hydroxybutyrate levels were also reduced only by combined maternal and postnatal HF diets (FE -1.09 ± 0.38 mmol/l, P = 0.008), and there were no significant differences in pairwise planned comparison tests.

Dependent variable	Significant effects	Fixed effect est.	P
Female plasma β-hydroxybutyrate	Maternal HF diet * Postnatal HF diet	-2.70 ± 0.89 mmol/l	= 0.006
Male plasma β-hydroxybutyrate	Maternal HF diet * Postnatal HF diet	-1.09 ± 0.38 mmol/l	= 0.008

Table 31 | Statistical results for Figure 35. HF, high-fat.

Expression of *Cpt1*, encoding carnitine palmitoyltransferase 1, the rate-limiting enzyme in mitochondrial fatty acid oxidation, was assessed in female offspring liver (Figure 36a, Table 32). Similar to β-hydroxybutyrate levels, combined maternal and postnatal HF diet was required to reduce *Cpt1* expression (logFE -0.28 ± 0.11, P = 0.014). There was no beneficial effect of metformin in HFm/HF offspring, yet Cm/HF offspring had significantly reduced *Cpt1* expression, suggesting a negative effect of metformin treatment in lean pregnancy on offspring who are subjected to postnatal HF diet. Relative mitochondrial copy number (Figure 36b, Table 32) was reduced by maternal HF diet (logFE -0.083 ± 0.038, P = 0.046) but unaffected by postnatal HF diet. Notably, maternal metformin treatment increased mitochondrial number in offspring lean dams (logFE 0.169 ± 0.041, P = 0.002), yet there was no effect in offspring of HF dams. Expression of *Ppara* (Figure 36c, Table 32), a PPAR family member highly expressed in the liver and playing a key role in regulation of fatty acid uptake and β-oxidation[410], was downregulated by maternal HF diet (logFE -0.09 ± 0.03, P = 0.012) and by the broad effect of metformin treatment (logFE -0.09 ± 0.04, P = 0.039). There was no specific effect of postnatal HF diet on expression. In planned comparison tests, the pairwise effect of metformin on *Ppara* expression was significant in Cm/HF vs C/HF offspring and HFm/C vs HF/C offspring, demonstrating an effect of metformin independent from

postnatal HF diet and indicating that the broad effect of maternal HF diet in reducing expression was the result of decreased expression in HFm/C, and not HF/C, offspring.

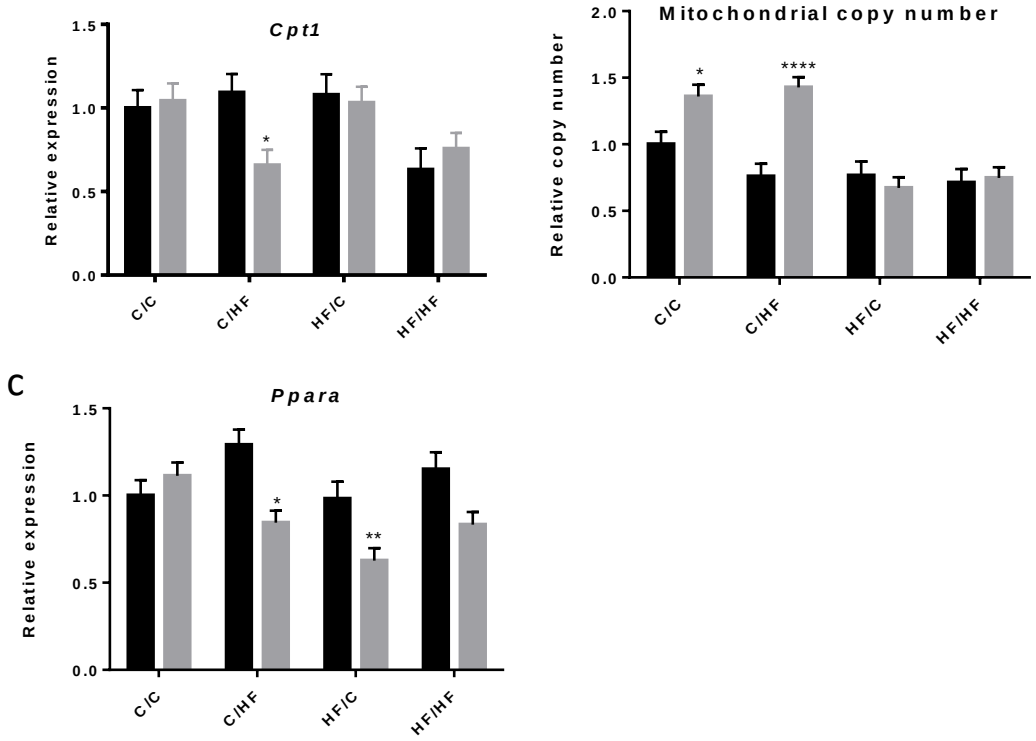

Figure 36 | **Hepatic expression of genes related to fatty acid oxidation in female offspring**. **a**. Hepatic mRNA levels of *Cpt1*. **b**. Hepatic mRNA levels of *Ppara* in adult female offspring. **c**. Hepatic mitochondrial copy number in adult female offspring. Gene expression data are non-logged predicted means ± SEM yet statistics were performed on logged data. Significant main effects and interactions are discussed in the main text. *, P < 0.05; **, P < 0.01;****, P < 0.0001 vs respective non-metformin group in planned comparison tests. C/C, n = 6 (from 5 litters); Cm/C, n = 8 (from 4 litters); C/HF, n = 6 (from 4 litters); Cm/HF, n = 10 (from 5 litters); HF/C, n = 5 (from 5 litters); HFm/C, n = 9 (from 5 litters); HF/HF, n = 5 (from 5 litters); HFm/HF, n = 9 (from 5 litters).

Dependent variable	Significant effects	Fixed effect est.	P
Cpt1	Maternal HF diet * Postnatal HF diet	-0.28 ± 0.11 logAU	= 0.014
Mitochondrial copy number	Maternal HF diet Metformin	0.083 ± 0.038 logAU 0.169 ± 0.041 logAU	= 0.046 = 0.002
Ppara	Maternal HF diet Metformin	-0.09 ± 0.03 logAU -0.09 ± 0.04 logAU	= 0.012 = 0.039

Table 32 | Statistical results for Figure 36. AU, arbitrary units; HF, high-fat.

Levels of circulating lipids in male and female offspring were measured to assess whether delivery to the liver might be altered (Table 33). Total cholesterol level was increased by postnatal HF diet in both females (FE 1.04 ± 0.21 mmol/l, P < 0.0001) and in males (FE 1.77 ± 0.32 mmol/l, P < 0.0001). There were no effects of metformin or maternal HF diet, although there was a trend

towards decreased total cholesterol levels in planned comparison tests in HFm/HF compared with HF/HF females (P = 0.07).

Table 33 | **Plasma lipid levels in female and male adult offspring groups.** Only total plasma cholesterol levels were significantly affected, and only by postnatal HF diet (right-most column). No pairwise planned comparisons were significant, although HFm/HF female total cholesterol levels trended towards a reduction compared with HF/HF females (P = 0.07). All values predicted means ± SEM. Females: C/C, n = 5 (from 4 litters); Cm/C, n =5 (from 4 litters); C/HF, n = 5 (from 4 litters); Cm/HF, n = 5 (from 5 litters); HF/C, n = 5 (from 3 litters); HFm/C, n = 5 (from 5 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 5 litters). Males: C/C, n = 5 (from 3 litters); Cm/C, n = 5 (from 5 litters); C/HF, n = 5 (from 2 litters); Cm/HF, n = 5 (from 4 litters); HF/C, n = 5 (from 4 litters); HFm/C, n = 5 (from 3 litters); HF/HF, n = 5 (from 4 litters); HFm/HF, n = 5 (from 4 litters). C, chow; FFA, free fatty acid; HF, high-fat; m, metformin; TAG, triglyceride.

Females	C/C	Cm/C	C/HF	Cm/HF	HF/C	HFm/C	HF/HF	HFm/HF	Significant effects
FFA (mmol/l)	1.97 ± 0.36	1.90 ± 0.36	1.96 ± 0.36	1.83 ± 0.36	1.85 ± 0.36	1.57 ± 0.37	1.66 ± 0.36	1.83 ± 0.36	None
Total cholesterol (mmol/l)	2.30 ± 0.30	1.97 ± 0.30	2.52 ± 0.29	3.40 ± 0.29	1.87 ± 0.31	1.98 ± 0.28	3.60 ± 0.30	2.80 ± 0.29	Postnatal HF (FE 1.04 ± 0.21, P < 0.0001)
TAG (mmol/l)	1.47 ± 0.29	1.08 ± 0.29	1.23 ± 0.29	1.61 ± 0.29	1.27 ± 0.29	1.07 ± 0.30	1.35 ± 0.29	1.34 ± 0.29	None

Males	C/C	Cm/C	C/HF	Cm/HF	HF/C	HFm/C	HF/HF	HFm/HF	Significant effects
FFA (mmol/l)	1.34 ± 0.18	1.45 ± 0.17	1.10 ± 0.20	1.42 ± 0.18	1.29 ± 0.16	1.45 ± 0.16	1.14 ± 0.16	1.29 ± 0.16	None
Total cholesterol (mmol/l)	2.93 ± 0.56	2.46 ± 0.54	3.39 ± 0.60	4.39 ± 0.57	2.73 ± 0.50	2.73 ± 0.50	4.60 ± 0.50	5.08 ± 0.51	Postnatal HF (FE 1.77 ± 0.32, P < 0.0001)
TAG (mmol/l)	0.98 ± 0.18	1.22 ± 0.17	1.04 ± 0.20	1.19 ± 0.18	1.25 ± 0.16	0.92 ± 0.16	0.94 ± 0.16	1.22 ± 0.17	None

7.4 Discussion

Disordered hepatic lipid metabolism is a key characteristic of NAFLD. Excessive hepatic lipid accumulation occurs as a result of increased hepatic fatty acid uptake, increased *de novo* fatty acid synthesis, or reduced oxidation of fatty acids[430]. Offspring of mothers who are obese have been shown to store excess hepatic lipid in early-life[68], and this finding persists into adulthood[67]. The data reported here are the first to show that an intervention during pregnancy, metformin, can alter lipid accumulation and composition in the offspring liver, in a manner dependent on the maternal diet and the sex and postnatal diet of the offspring. Specifically, metformin exerts some beneficial effects in female offspring of obese dams, including decreasing CE accumulation, although total lipid levels were not altered. Hepatic expression of *Ppara*, *Pparg* and *Srebp1* was reduced by metformin treatment in these offspring, and fasted plasma levels of β-hydroxybutyrate were rescued in HFm/HF animals compared with HF/HF offspring, indicating markedly improved ability to oxidise hepatic fats. By contrast, metformin given to lean dams exerted detrimental effects (including increased total hepatic lipid levels) in offspring that received a postnatal HF diet; these effects were most pronounced in male offspring, although worrying trends towards increased expression of prolipogenic genes were seen in Cm/HF females.

That maternal obesity increases hepatic lipid accumulation has now been documented in human neonates and in animal models[66]. In the data presented here, maternal obesity did not independently increase the level of total assayed lipid in either sex offspring, although there were strong trends in females ($P = 0.057$) and males ($P = 0.051$), respectively. In general, TAG represented the largest proportion of hepatic lipids in males and females, although female CE levels were higher than TAG in HF/HF and HFm/HF offspring.

In females, only levels of CE were significantly increased by maternal HF diet, suggesting a priming of either the formation of CEs (such as increased activity of hepatic ACAT) from free cholesterol, decreased levels of free cholesterol providing substrate for CE formation, or decreased de-esterification (such as decreased activity of cholesterol ester hydrolase). Min *et al.* have shown that hepatic free cholesterol accumulation without increased CE is a hallmark of NAFLD in humans[431], in contrast to the parallel increases in CE and free cholesterol seen in animal models[432]. As such, this finding is of limited direct applicability to humans. However, metformin treatment did reduce CE accumulation, suggesting an improvement of the mouse NAFLD phenotype, potentially by reversal of one of the above mechanisms. Measurement of hepatic free cholesterol levels and activity of the cholesterol synthesizing enzyme HMG-CoA reductase would be an interesting further experiment, as a potential priming effect of metformin on free cholesterol production could be beneficial in human NAFLD.

There were no specific effects of maternal HF diet on hepatic TAGs, PCs or NEFAs in female offspring; another group investigating the effect of maternal obesity on rat offspring hepatic lipids at 3 months of age found significant maternal effects, although their study was apparently powered (albeit with no consideration of litter-of-origin) to detect smaller between group variance[432]. Neither maternal diet nor metformin treatment altered the ratio of total n-3 to n-6 PUFAs in the present study.

In male offspring, metformin significantly increased total hepatic lipid levels, irrespective of maternal diet. This finding was largely the result of a dramatic increase in lipid levels in HFm/HF offspring compared with HF/HF animals. When broken down by lipid fraction, TAGs were predominantly responsible for this increase, of which SFAs (2.1-fold) and MUFAs (2.5-fold) were significantly increased vs HF/HF offspring, although there were trends towards the increase of both n-6 and n-3 PUFAs. Therefore, metformin seems to prime increased TAG accumulation in these animals, relatively independently of other lipid fractions and entirely dependent on sex.

DNL is upregulated in patients with NAFLD and contributes a substantial portion of fatty acid to the hepatic lipid pool[18]. Ratios between the products of enzymatic reactions and a relatively stable fatty acid can be used to infer enzymatic activity, although comparisons should be avoided between groups eating different postnatal diets. In this model, total DNL ratio in female offspring was modestly increased by maternal diet, indicating a contribution of DNL to the total hepatic lipid pool in these animals. Prior work has shown that upregulated expression of DNL enzymes is primed by maternal obesity[70,389]. Here, metformin treatment reduced DNL ratio in female offspring of obese dams, indicating lower DNL activity. Δ9 SCD desaturation index, the ratio of oleic acid to the saturated fatty acid precursor stearic acid, was also increased by maternal HF diet, yet there was no effect of metformin treatment. The terminal product of DNL is palmitic acid, elongation of which derives stearic acid, which in turn is desaturated by SCD1 to oleic acid. As a result, increases in DNL can provide increased substrate, via elongation, for desaturation, linking both indices. Increases in both DNL and desaturation indices have been observed in individuals with NAFLD[418], and increased desaturation index and SCD1 protein levels have been shown in chow-fed offspring of obese dams compared with offspring of lean dams[428]. However, the association between elevated Δ9 desaturation index and hepatic lipid content in humans is controversial, with some studies finding a positive relationship and others a negative relationship, depending on the lipid fraction analysed and the adiposity of the patients[433]. Li *et al.* showed that mice lacking SCD1 fed a methionine–choline-deficient diet had increased hepatic injury compared with wild-type animals, an effect mitigated when they were fed with MUFAs, implying a protective function of SCD1 in converting SFA to MUFA[62].

Hepatic expression of *Srebp1*, which regulates transcription of the key lipogenic enzymes FAS, ACC1 and ACLY, was only increased by postnatal HF diet; however, maternal metformin in HF dams decreased *Srebp1* expression in offspring. Downstream of *Srebp1*, levels of *Fas*, *Acc* and *Acly* were unaltered by maternal diet or metformin, suggesting that alterations to *Srebp1* mRNA levels were not associated with altered transcription of lipogenic gene targets, and that mRNA levels of these lipogenic genes are not related to the metformin-mediated reduction in DNL. Thus, the observed reduced DNL index could be caused by: 1) altered delivery of substrate for DNL (that is, acetyl-CoA); 2) allosteric regulation of ACC1 via accumulation of inhibitory molecules such as malonyl-CoA and fatty acyl CoA; 3) alterations in enzyme activities or protein expression not manifested in transcript levels[434]. These alternative mechanisms were not assessed in this study, providing a further research avenue.

Relevant hepatic TAG synthesis enzyme expression has not yet been assessed in male offspring in this model, and the markedly different phenotypic response to metformin in the HFm/HF and HF/HF male groups compared with equivalent female offspring groups precludes drawing any inferences from female gene expression data. DNL ratio was significantly increased in HFm/HF compared with HF/HF males, indicating increased hepatic DNL might be at least partially responsible for the increased lipid levels observed. Δ9 SCD desaturation index was also substantially increased in HFm/HF males vs HF/HF offspring. Given that HFm/HF males in this study displayed increased amounts of total MUFA and SFA, increased desaturation activity might be a protective response to increased synthesis of SFA via DNL. However, this hypothesis awaits more direct measurement of SCD1 expression and activity.

Fatty acid transport is another mechanism which can influence hepatic lipid levels. No differences were observed in circulating lipid levels in adult offspring as a result of metformin treatment, suggesting that altered hepatic lipid levels are not the result of altered delivery from circulation. In contrast to these results, Salomaki *et al*. showed reduced plasma TAG and total cholesterol levels in males of obese dams treated with metformin, compared with male offspring of obese untreated dam[255]. This different result could be related to differences in the offspring phenotype compared with male offspring of obese dams reported in this thesis; for instance, males in the Salomaki *et al.* study were only exposed to postnatal HF diet from 10 weeks of age, for 6 weeks. Thus, any beneficial effect of metformin on plasma total cholesterol and TAG levels in studies conducted in this thesis might be masked by the much longer postnatal HF diet duration (27 weeks) or the effect of increasing age. Metformin could be priming alterations to the HDL to LDL cholesterol ratio, which was not assessed in this thesis; low levels of HDL relative to LDL are a feature of the metabolic syndrome and observed in patients with NAFLD[30]. Levels of *Lpl*, which is not normally expressed in adult liver[435], were increased by combined maternal and postnatal HF

diets, and significantly reduced by metformin treatment in offspring of HF dams. LPL catalyses the breakdown of TAGs from VLDL and chylomicrons, allowing uptake by peripheral tissue (predominantly adipose tissue and skeletal muscle)[436]. Aberrant expression in the liver could result in increased fatty acid uptake, leading to inappropriate lipid accumulation. Hepatic *LPL* has been shown to be upregulated in human patients with fatty liver[437]. Hepatic *Fatp2* levels were not increased by maternal or postnatal HF diets, but did decrease when maternal and postnatal HF diets were combined. This response might represent an adaptation to decrease fatty acid uptake in a compensatory manner, given that HF/HF and HFm/HF offspring have the highest levels of hepatic lipid accumulation. Knock-down of FATP2 in cultured hepatocytes reduces fatty acid uptake, whereas forced upregulation increased uptake, and knockdown of FATP2 in mice has been shown to reverse hepatosteatosis[438].

Finally, hepatic fatty acid oxidation represents a major route of fatty acid removal from the liver. Fasted plasma levels of β-hydroxybutyrate were dramatically decreased (3.85-fold) in female offspring only when postnatal and maternal HF diets were combined. β-hydroxybutyrate, along with acetoacetate, is a ketone produced from fatty acids by hepatic β-oxidation during fasting to provide energy for extrahepatic organs. Reduced fasting levels of this ketone body thus indicate an inability to oxidize fatty acids in response to fasting in female HF/HF animals, recapitulating the results of Bruce *et al.*[70]. Maternal metformin treatment strikingly rescued β-hydroxybutyrate levels in HFm/HF female offspring, although levels of *Cpt1*, which were decreased in HF/HF offspring, were not improved in HFm/HF animals. Thus, these data suggest a mechanism by which metformin promotes β-hydroxybutyrate formation in HFm/HF females without modulating *Cpt1* expression in the liver. There were no differences in mitochondrial copy number between the HFm/HF and HF/HF females, implying either that CPT1 activity or protein abundance does not reflect mRNA levels, and/or that other enzymes responsible for mitochondrial β-oxidation are altered. Bruce *et al*. showed that mitochondrial oxidative capacity is impaired by maternal HF diet and especially in HF/HF females[269]. As the purpose of β-oxidation is to provide ATP, the process is intrinsically linked to oxidation levels of other substrates, including glucose, and thus to electron transport chain activity. For instance, complex I of the electron transport chain is required to re-oxidize NADH; if this re-oxidation is impaired, enzymes requiring NADH that perform β-oxidation (3-hydroxyacyl-CoA dehydrogenases) will be inhibited[71]. Crucially, Bruce *et al.* showed that complex I activity was markedly decreased in HF/HF offspring, thereby providing a potential regulatory mechanism by which metformin treatment could alter mitochondrial fatty acid metabolism without altering levels of *Cpt1*[269]. Experiments to determine the activity of the hepatic mitochondrial electron transport chain as a result of metformin in maternal obesity are

therefore required, as is assessment of expression and activity of key enzymes involved in β-oxidation.

In male offspring, β-hydroxybutyrate levels were again markedly reduced only by combined exposure to maternal and postnatal HF diet, yet there was no effect of metformin in alleviating the reduction. This finding reinforces the existence of pronounced sex differences in the effects of metformin on developmental priming. Suppressed β-oxidation in HF/HF and HFm/HF males, and the inability of metformin to upregulate it (as seen in female HFm/HF offspring), could underlie the disparity between total hepatic lipids in male HFm/HF offspring compared with female HFm/HF offspring.

Interestingly, hepatic levels of *Ppara*, a master regulator of β-oxidation-related gene transcription in the fasted state, were reduced in HFm/C vs HF/C females, and there was a strong trend towards reduced expression in HFm/HF vs HF/HF offspring also. Amongst other targets, *Ppara* directly regulates transcription of the β-oxidation enzymes *Mcad, Lcad* and *Vlcad*, although expression of these targets was not assessed. Increased levels of RXR, which heterodimerizers with PPARα to initiate transcription of target genes, or increased levels of PPARα agonists might underlie the indicated increase in hepatic B-oxidation in the absence of *Cpt1* or *Ppara* gene expression increases. Levels of the n-3 PUFAs DHA or EPA, which are potent PPARα agonists[410], were not significantly increased in the total lipid pool, although the PC subfraction was significantly enriched for n-3 PUFAs in HFm/HF offspring compared with HF/HF animals.

HF/HF offspring had increased hepatic expression of *Pparg* as a result of both maternal and postnatal HF diets. This gene is typically expressed at low levels in the liver, instead being a key gene regulating adipogenesis. Rodents show increased *Pparg* expression in the liver as steatosis increases, but this increase is not observed in humans with NAFLD[439]. *Ob/ob* mice are resistant to hepatic steatosis when *Pparg* is also knocked out in the liver, although they develop increased insulin resistance[440]. Thus, the translational relevance of the observed decrease of *Pparg* expression in HFm offspring compared with HF offspring is unknown. Tong *et al.* showed in a mouse model that offspring skeletal muscle *Pparg* expression, which was increased by maternal obesity, was reduced by maternal metformin treatment[145]. *Acc1* is a known target of *Pparg*, yet expression was not increased in HF/HF offspring; aside from differences between protein and transcript levels, altered levels of PPARγ agonists, reduced levels of transcriptional coactivators or the PPARγ heterodimeric partner RXR might be responsible for the lack of indirectly observed PPARγ transcriptional activity[439].

Although the effect of metformin treatment on liver lipid composition in fetal offspring of obese dams has been assessed, no previous work has assessed lipid profiles in adult offspring of lean

dams given metformin. Here, both female and male Cm/HF offspring showed trends towards increased hepatic TAG and CE accumulation compared with respective C/HF groups. In females, these trends towards increased lipid levels in Cm/HF vs C/HF offspring were also observed in SFAs and MUFAs. However, in males, increased MUFA levels in Cm/HF animals were significantly increased vs C/HF animals. Thus, Cm/HF offspring in both sexes displayed a trend towards mild increases in hepatic lipids.

DNL ratio in Cm/HF females was increased relative to C/HF offspring, and was associated with concomitant trends towards increased hepatic *Srebp1* and *Fas* expression. Given the dissociation between expression of hepatic lipogenic genes and DNL index in offspring of HF dams, caution should be urged linking DNL ratio in Cm/HF offspring with mRNA levels of lipogenic genes. However, models of maternal undernutrition, of which metformin in lean pregnancy seems to mimic (Chapter 4), result in primed hepatic DNL in offspring. This priming includes upregulation of SREBP1c at both protein and transcript levels and downstream targets including ACC1 and FAS[441]. Desaturase index was also increased in Cm/HF vs C/HF offspring. Males also displayed trends towards increased DNL and desaturation indices. Whether this apparent upregulation of fatty acid synthesis is associated with hepatic lipogenic gene expression remains to be determined.

Fasting plasma levels of β-hydroxybutyrate were significantly decreased in Cm/HF female offspring compared with the C/HF group, suggesting an inability to effectively oxidise hepatic lipids in response to fasting. Interestingly, this finding was also associated with decreased hepatic *Cpt1* expression in this group, as well as increased numbers of hepatic mitochondria. The effects of metformin during lean pregnancy on offspring hepatic mitochondrial copy number are unclear. Activation of AMPK via metformin increases mitochondrial biogenesis acutely in cultured human umbilical vein endothelial cells[231]. AMPK activation via other agents, such as 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), also increases mitochondrial biogenesis, and metformin is well-appreciated to activate AMPK in hepatocytes[442]. Thus, acute metformin treatment in lean dams, which likely leads to increased AMPK activation compared with treatment in obese dams, probably induces mitochondrial biogenesis in hepatocytes, although it remains unclear whether this effect is persistent and results in the higher numbers of mitochondria observed in Cm/HF offspring. *Cpt1* expression, as described earlier, is under the control PPARα; here, levels of *Ppara* mRNA were reduced in Cm/HF vs C/HF animals, suggesting a potential transcriptional mechanism leading to repression of *Cpt1*. Interestingly, there was no reduction in β-hydroxybutyrate levels in male Cm/HF offspring compared with C/HF animals, suggesting intact fatty acid oxidation capacity in spite of the trends towards increased total lipids in this offspring group.

In contrast to the offspring of HF dams, mRNA levels of *Lpl* and *Fatp2* were unaltered in Cm/HF vs C/CHF offspring, suggesting the absence of priming effects in lean metformin-treated-dams on transcriptional regulation of fatty acid transporters. Whether these mRNA levels are reflective of protein levels or enzymatic activity has not been determined here, but they do hint that altered hepatic lipid homeostasis might occur in Cm/HF offspring independent of alterations to proteins involved in fatty acid uptake.

In summary, the data reported in this chapter show that maternal HF diet promotes offspring hepatic lipid accumulation via a number of mechanisms, dependent on offspring sex. Female offspring of HF dams show increased DNL, desaturase index, and an impaired ability of oxidize hepatic lipids during fasting. Liver transcript levels of *Lpl* are upregulated and *Fatp2* downregulated, indicating disordered fatty acid uptake. However, metformin treatment in HF dams reduces DNL, and improves the lipid oxidative ability of HFm/HF female offspring, through mechanisms that are not yet apparent. In the preclinical setting, use of PPARα agonists (for example, fibrates) alleviates hepatic steatosis in part via upregulation of β-oxidation[443], highlighting the potential benefit of this effect. In males, HFm/HF offspring phenotype was substantially different from the female phenotype, showing increased total hepatic TAGs, driven at least in part by increased DNL and an inability of metformin to rescue fatty acid oxidation activity. Offspring of lean, metformin-treated dams showed patterns of increased hepatic lipid accumulation, irrespective of sex. In females, this finding was accompanied by increased DNL and decreased ability to remove lipids via oxidation.

Together, the increased levels of hepatic lipids resulting from metformin treatment, in Cm/HF male and female offspring and in HFm/HF male offspring, requires further exploration given their potential detrimental effects on metabolic health. Particularly worrying is the finding that adult male offspring of obese dams given an HF postnatal diet show markedly increased hepatic lipid deposition, which has potential ramifications for male children born to obese mothers given metformin during pregnancy. The source of these lipids prompts further investigation; for instance, TAGs are enriched relative to CEs, suggesting preferential storage and priming of lipogenic enzyme activity. It would be interesting to measure levels of ceramides and DAGs, which exert significant lipotoxic effects and are increased in patients with NAFLD[61]. Moreover, free cholesterol, hepatic accumulation of which is strongly associated with NAFLD, was not measured. Whether these lipid fractions are also increased in HFm/HF males is unknown.

Chapter 8: Discussion

8.1 Introduction

Maternal obesity is a substantial and growing public health issue. In the UK, approximately 1 in 5 women enter pregnancy obese[97], and this number seems to be increasing. In the US, prevalence of obesity in women aged 20–39 years was approximately 32% in 2011, up from 25% in the mid-2000s and about 15% in 1990. With increasing globalization, greater numbers of women of reproductive age are obese in lower and middle income countries. For instance, 0.8 million women were obese in India in 1975; by 2014, 20 million women were estimated to be obese[444].

The short-term risks to mother and child in pregnancies complicated by obesity are by now well-appreciated. However, the past two decades has seen growing attention paid to the long-term effects of maternal obesity on the offspring. One of the foremost risks, in terms of volume of research and in terms of healthcare burden, is increased offspring adiposity and associated metabolic disease, including NAFLD. Despite the increasing evidence base linking maternal obesity to these outcomes in offspring, research into potential interventions has been limited until the past few years. Clinical trials of lifestyle modifications have failed to have the desired effect on neonatal outcomes (such as incidence of large-for-gestational age), despite often reducing gestational weight gain and improving maternal diet[66,190]. As a result, new interventional approaches are required.

Metformin represents an attractive pharmaceutical option for several reasons: 1) it is generic, cheap and widely available, facilitating global use and exerting a low burden on healthcare systems; 2) it has diverse antihyperglycaemic, anti-inflammatory and antidyslipidaemic properties; and 3) it has demonstrable safety during pregnancy[241]. In addition, metformin crosses the placenta, enabling it to exert effects directly in fetal tissue. As a result of these characteristics, metformin has been trialled as an initial alternative to insulin in women with gestational diabetes[198], and as an intervention in pregnant women with obesity[243,377].

8.1.1 Requirement for a model of maternal metformin treatment

Initial results from studies in obese human pregnancy did not suggest an effect of metformin on neonatal outcomes. In the MiG trial, which compared metformin with insulin for the control of gestational diabetes, maternal and neonatal outcomes were similar, yet there were suggestions that offspring at 2 years-of-age had greater subcutaneous fat, with no change in total adiposity, if given metformin[239]. In the EMPOWaR trial, metformin treatment (up to 2.5g per day) during pregnancy in women who were obese (BMI >30kg/m^2) without gestational diabetes did not reduce offspring birth weight or prevent gestational diabetes development[243]. In the MOP trial, metformin (up to 3g per day) or placebo was given to women with a BMI > 35kg/m^2 during

pregnancy[244]. Despite reducing gestational weight gain and lowering incidence of preeclampsia, there was no effect on offspring birth weight.

Thus, these trials establish that metformin treatment prescribed during early obese pregnancy (12–18 weeks) at high doses is not able to reduce offspring birth weight. However, whether the effects of metformin treatment manifest in the offspring in later life are not determined by these results, and the children included in these studies will require follow-up for several decades to assess long-term disease risk. Even then, measurements in study participants are limited to largely non-invasive interrogations, and no investigation of fetal or neonatal parameters is possible, precluding detailed assessment of mechanisms that might underlie potential long-term effects. An animal model of maternal obesity, treated with a human-relevant dose of metformin, is therefore required to determine whether metformin primes alterations to the offspring phenotype in adulthood.

8.1.2 Rationale for employment of this model

The maternal obesity model employed in this thesis is the same model used to explore the effect of maternal overnutrition during pregnancy on offspring NAFLD severity; offspring of obese dams showed marked priming of NAFLD severity when mothers are untreated[70]. This model uses a high-fat, obesogenic diet (containing 45% kcal fat (predominantly from lard), 35% kcal carbohydrate (from rice starch and sucrose) and 20% kcal protein (from casein), with additional vitamins and minerals) administered for 6 weeks to proven-breeder females. As demonstrated in Chapter 4, after 6 weeks of diet feeding, HF dams are heavier than C dams, and show impaired fasting blood glucose and glucose intolerance. Thus, the model reflects the increased maternal adiposity and excess nutrient intake observed in women who are obese during pregnancy, and recapitulates glucose intolerance that is a common feature of obese pregnancy. In the UK, NICE recommends that women who are obese are screened for gestational diabetes (defined by elevated 2 h blood glucose after an oral glucose tolerance test) at 24–28 weeks of pregnancy, leaving a large window for elevated blood glucose to remain undiagnosed and untreated. A model of human maternal obesity that does not recapitulate maternal glucose intolerance could therefore be considered unrepresentative of the clinical reality for many women worldwide.

Dose of metformin used in this study was 250 mg/kg, similar to that used by several other mouse models of maternal metformin treatment[253,255,256], as such a dose produces blood and amniotic fluid concentrations in the range observed in human treatment (Chapter 4). Treatment commenced at conception, whereas treatment initiation in the MOP and EMPOWaR studies began at time of booking, typically 12–18 weeks into pregnancy, by logistical necessity. The earlier

treatment commencement used in this thesis could be expected to increase offspring exposure to the drug relative to beginning treatment later, including increasing exposure during early critical development windows. The treatment start-time used in the MOP, EMPOWaR and lifestyle intervention studies might be too late to alter the course of maternal-obesity-mediated effects on neonates, and has been advanced as reason for their ineffectiveness[190,445]. Interventions to mitigate maternal weight gain that are delivered earlier in pregnancy do better than those delivered later in pregnancy, raising the question of whether interventions should be introduced as early as possible, perhaps even before conception[153]. This prophylactic approach of metformin therapy preconception is used to improve fertility in women with PCOS, with beneficial maternal and neonatal outcomes, especially when metformin is continued throughout pregnancy[446].

Finally, in the model described in this thesis, metformin treatment was continued after birth and through lactation, as in the model employed by Tong *et al*.[145]. The basis for this decision is that the maturation status of key metabolic organs in the mouse neonate in the days after birth is approximately equivalent to that of human fetuses in the weeks before birth, particularly the liver[447]. Development of adipose tissue, which occurs in primates primarily during the final third of gestation[448], occurs later in rodents, with substantial postnatal development[449]. Thus, the lactation period is important in the context of developmental priming in animal models and in humans, in which the benefits of breastfeeding are well-appreciated. Although the dosage of metformin received by the neonate through milk is lower than that received by the fetus *in utero*[450], metformin might still exert effects on the offspring via alteration of milk composition as a result of improved maternal metabolism (for instance, higher levels of adiponectin and lower levels of leptin in maternal plasma)[451], by modulating microbial colonization[392], or interactions between both factors[452].

The model used in this thesis differs from recently reported mouse models of maternal metformin treatment during obesity in several ways. In the model used by Salomaki *et al.*, mothers were switched from HF diet to chow diet during lactation, disturbing the obesogenic insult[255]. Moreover, metformin was only given from E0.5 to E17.5, and postnatal HF diet in offspring was commenced not from weaning but from postnatal week 11. Thus, this model does not assess the effect of continuous obesogenic diet exposure or maternal metformin treatment on maternal priming. In the mouse model used by Harris *et al.*[256], metformin was not given to lean dams, giving no ability to assess the phenotype of offspring of this group, although they used a similar dose of metformin (300 mg/kg dissolved in drinking water) as used in this study. The models of Harris *et al*.[256] and Desai *et al.*[268] both also only assessed fetal outcomes. The model of Tong *et al.*[145] is most similar to the model used in this thesis; obese female mice were given metformin at a concentration of 2 mg/ml (1.75 mg/ml was used in this thesis) for the duration of pregnancy and

lactation, although lean dams were not given metformin. Moreover, only skeletal muscle was assessed.

8.2 Overview of findings

The first major novel finding of this thesis is that maternal metformin treatment, in an established mouse model of maternal obesity, decreases severity of NAFLD in female offspring only. Histologically assessed NAFLD, which is exacerbated by maternal diet, was reduced in severity by metformin in these animals. This finding was accompanied by reduced expression of pro-inflammatory markers in HFm/HF animals, yet no reduction in HFm/C offspring, suggesting a differential NAFLD phenotype as a result of postnatal HF diet.

The second major novel finding reported here is that maternal metformin treatment dramatically exacerbates NAFLD severity in HF-fed offspring born to lean dams. In this case, the effects of metformin were seen in both male and female offspring, and consisted of increased hepatic lipid deposition, inflammation and markers of increased DNL.

Finally, these data demonstrate the marked sexual dimorphism observed in priming of offspring physiology and disease by diet, obesity and metformin, and highlight the importance of assessing both sexes to obtain a complete picture.

8.2.1 Effect of metformin in dams and fetuses

Findings from this study in dams (Table 34) and fetuses (Table 35) are summarized in tables below.

Table 34 | **Summary of the effects of metformin in dams at E16.5.** nc, no change in parameter; ↑, increase of parameter; ↓, decrease of parameter; C, chow diet; FBG, fasting blood glucose; HF, high-fat diet.

	Effect of metformin	
Parameter	**C dams**	**HF dams**
Body weight	**nc**	**nc**
FBG	**nc**	**nc**
Glucose tolerance	**nc**	**nc**
Litter size	**↓**	**↑**

Table 35 | **Summary of the effect of metformin treatment on fetuses at E16.5.** nc, no change in parameter; ↑, increase of parameter; ↓, decrease of parameter; C, chow diet; BG, blood glucose; HF, high-fat diet.

	Effect of metformin	
Parameter	**Fetuses from C dams**	**Fetuses from HF dams**
Body weight	↓	↓
Placenta weight (% fetal weight)	↑	↑
Liver weight	↓	↓
Liver weight (% fetal weight)	**nc**	**nc**
Unfasted BG	↑	↑
Hepatic mRNA		
Sirt1	↑	↑
Sirt3	**nc**	**nc**
Pgc1a	**nc**	**nc**
Nrf1	**nc**	**nc**
Tfam	↑	**nc**

Although the acute effects of metformin on fetal tissue in rodent models have been studied in other publications[256,268], the data reported in this thesis are the first to link fetal outcomes, described in Chapter 4, with a detailed characterization of the adult offspring metabolic and hepatic phenotype, described in Chapters 5, 6 and 7.

8.2.1.1 Metformin does not significantly alter dam glucose homeostasis

Although the first hypothesis described in Chapter 2 postulated that metformin would improve maternal glucose homeostasis, the data presented show that metformin did not significantly improve maternal metabolic parameters at E16.5. Body weight, fasting blood glucose, glucose tolerance and random non-fasted insulin levels were all unaltered. This lack of efficacy is therefore similar to those reported in the MOP[244] and EMPOWaR[243] studies, and Salomaki *et al.* also reported no effect of metformin on bodyweight at E17.5[255]. They did observe a significant beneficial effect of metformin on maternal HF dam glucose tolerance, yet this was measured at E11.5–12.5, when gestation-related insulin resistance is lower and thus there is greater scope for observing a beneficial effect of metformin[453]. Thus, metformin may exert beneficial metabolic effects in this model that are no longer observed at E16.5 due to increased insulin resistance. Moreover, there was a trend in this study towards the reduction of maternal plasma CRP level, a

finding also reported in the EMPOWaR study[243]. This data is suggestive that metformin reduces markers of systemic inflammation that are otherwise increased by obesity. Whether this inflammation, and it's reduction by metformin, is related to the priming of offspring in this study is unclear; Desai *et al.* found no effect of metformin on maternal plasma IL-6, IL-1β, CCL2 or TNF levels, none of which were measured in this study, and they did not assess CRP levels[268]. Standard errors for fixed effect estimates of the effect of metformin on glucose homeostasis parameters were relatively large, and may have masked a significant beneficial effect of metformin. There were only four dams in each of the groups for these parameters, reducing statistical power.

8.2.1.2 Metformin reduces fetal body weight but rescues litter size reductions

Fetuses born to metformin-treated dams were smaller than those born to non-treated dams of either diet group, suggesting that metformin restricts growth at this time point via an undetermined mechanism. The data for HFm fetuses are in contrast to the first hypothesis described in Chapter 2, which postulated that metformin would mitigate the effects of maternal obesity on fetal growth at E16.5. However, the reduced fetal body weight and liver size in Cm fetuses is in support of a hypothesized growth restriction effect in these offspring, especially when viewed in the context of adult Cm offspring phenotypes.

It may be that metformin treatment earlier in pregnancy suppresses maternal blood glucose and fetal nutrient delivery to such a degree as to induce intrauterine growth restriction at E16.5, followed by 'catch-up' growth in the final days of gestation and prior to weaning, at which point there are no differences in body weight between metformin and non-metformin treated offspring. Interestingly, catch-up growth is typically associated with exacerbated metabolic dysfunction, typified by increased adiposity when offspring are given a postnatal obesogenic insult, which was not observed in adult offspring of HF dams of either sex[454,455], suggesting that this reduced fetal body weight in HFm animals is not detrimental to the adult offspring phenotype. Alternatively, whether altered placental function is the route of the negative effect of metformin on fetal body weight was not assessed in this study, and the effect of metformin on placental tissue is understudied; in their rat model of maternal obesity, Desai *et al.* showed no effect of metformin on placental weights or levels of TNF, which was the only examined inflammatory marker upregulated in obese placentas. Assessment of placental levels of inflammatory markers and transport proteins in placentas collected for this thesis is ongoing.

In the experiments reported in this thesis, metformin did rescue litter size in HF dams, introducing the possibility that the same (or greater) quantity of maternal resources are allocated over a larger number of fetuses, effectively distributing fetal biomass. On the basis of data showing altered placental:fetal weight ratio resulting from both maternal HF diet and metformin

treatment, placental nutrient transfer in this model and its association with fetal growth and adult offspring phenotype should be assessed in the future.

8.2.1.3 Metformin alters fetal hepatic gene expression

Metformin increased fetal hepatic expression of *Sirt1* and *Tfam*, with Cm fetuses showing approximately twofold increases in expression of both genes compared with C fetuses. Data reported in Chapter 7 show that hepatic mitochondrial copy number (which might be a downstream effect of increased SIRT1 activation mediated by upregulated TFAM activity[456]) was increased in female offspring of Cm dams (in both Cm/C and Cm/HF groups), suggesting primed and persistent increases in the number of hepatic mitochondria in these offspring. Interestingly, expression of *Sirt1* in adult female liver was unchanged between groups except for a marked (approximately twofold) elevation in Cm/C animals. This finding suggests that increased hepatic *Sirt1* expression might be primed in early life by metformin exposure in lean dams, yet suppressed upon exposure to a postnatal HF diet. Whether the increased mRNA expression of *Sirt1* at this stage in development in HFm offspring has any lasting effects is unclear, and would require analysis of protein expression and activity at fetal, early postnatal and adult time points. Increased hepatic expression of SIRT1 is associated with protection against hepatic steatosis via effects on fatty acid metabolism (including increased PPARα expression and β-oxidation[457] and inhibition of SREBP1c-mediated lipogenesis[329]) and reduced activation of pro-inflammatory cytokines[458]. These effects were observed in HFm offspring in adulthood, in the absence of increased *Sirt1* mRNA expression. It is possible that SIRT1 activity or protein abundance is increased in adult offspring HFm dams, explaining these effects; alternatively, upregulation of *Sirt1* only in early life might be sufficient to either prime expression of the above pathways, or to protect against the deleterious effects of early hepatolipotoxicity. Studies of maternal supplementation with resveratrol, a SIRT1 activator, during obese nonhuman primate pregnancy have shown that the compound reduces fetal hepatic lipid accumulation[459], yet long-term effects in this model have not been reported and resveratrol treatment was associated with unexplained pancreatic mass increase.

8.2.2 Long-term effects of metformin

Metformin exerted persistent effects on adult offspring phenotype. As these effects differed drastically depending on whether metformin was given to obese HF dams or lean C dams, the summary of these effects is split by dam diet. A summary of the effects of metformin on adult offspring are shown separated by sex in Table 36. The sex differences in response to metformin in offspring of obese dams are immediately apparent.

Table 36 | **Summary of significant metformin effects in male and female offspring of lean and obese dams**. nc, no significant change in parameter (trends P < 0.1 not shown)); ↑, significant increase of parameter; ↓, significant decrease of parameter; C, chow diet; CE, cholesterol esters; DNL, *de novo* lipogenesis; FBG, fasting blood glucose; HF, high-fat diet; HOMA-IR, homeostatic model assessment of insulin resistance; Mt, mitochondrial; MUFA, monounsaturated fatty acids; NA, not applicable (not measured); NAS, NAFLD activity score; NEFA, nonesterified fatty acids; PC, phosphatidylcholines; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAG, triglycerides.

Parameter	**Female offspring of obese dams**	**Male offspring of obese dams**	**Female offspring of lean dams**	**Male offspring of lean dams**
Adiposity				
Body weight	↓	nc	↑	nc
Body fat %	↓ (mostly HFm/C)	nc	↑	↑
Visceral adipose	↓	nc	↑	nc
VAT:SAT	nc	nc	nc	nc
iBAT	↓	nc	nc	nc
Cardiovascular				
SBP	nc	nc	nc	nc
DBP	nc	nc	nc	nc
Heart mass	nc	↓ (only HFm/HF)	nc	nc
Glucose homeostasis				
FBG	↓ (only HFm/HF)	nc	nc	nc
Glucose intolerance	↓	nc	↑	↑
Fasting insulin	↓ (only HFm/HF)	nc	nc	↑
HOMA-IR	↓ (only HFm/HF)	nc	nc	↑
Plasma inflammatory markers				
IFNγ	nc	**NA**	nc	**NA**
CRP	nc	**NA**	↑	**NA**
IL-6	nc	**NA**	nc	**NA**
IL-10	↓ (mostly HFm/C)	**NA**	↓ (mostly HFm/C)	**NA**
Liver-related measures				
Liver weight	↓	nc	↑	nc

Liver weight (% body weight)	↓ (only HFm/HF)	nc	nc	nc
NAS	↓	nc	↑	↑
NAS -inflammation	↓	↑	nc	↑
NAS – steatosis	nc	nc	nc	nc
NAS - ballooning	nc	nc	nc	nc
Steatosis %	nc	**NA**	↑	**NA**
Inflammation-related hepatic gene expression				
Ccl2	↓	**NA**	nc	**NA**
Il1b	nc	**NA**	nc	**NA**
Tlr2	↓	**NA**	nc	**NA**
Tlr4	nc	**NA**	nc	**NA**
Opn	↓ (only HFm/HF)	**NA**	nc	**NA**
Hepatic lipids				
Total hepatic lipid	nc	↑ (only HFm/HF)	nc	nc
TAGs	nc	↑ (only HFm/HF)	nc	nc
CEs	↓ (only HFm/C)	↑	nc	↑
PCs	nc	nc	nc	nc
NEFAs	nc	↓	nc	nc
SFAs	nc	↑ (only HFm/HF)	nc	nc
MUFAs	nc	↑ (only HFm/HF)	nc	↑
n-3 PUFAs	nc	↑ (only HFm/HF)	nc	nc
n-6 PUFAs	nc	nc	nc	nc
DNL index	↓	↑	↑	↑
Desaturation index	nc	↑	↑	↑
Lipid-homeostasis-related hepatic gene expression				
Srebp1	↓	**NA**	nc	**NA**
Fas	nc	**NA**	nc	**NA**
Acly	nc	**NA**	nc	**NA**
Acc1	nc	**NA**	nc	**NA**
Pparg	↓	**NA**	nc	**NA**

Lpl	↓ (mostly HFm/C)	**NA**	nc	**NA**
Fatp2	nc	**NA**	nc	**NA**
Markers of hepatic β-oxidation				
Plasma β-hydroxybutyrate	↑ (only HFm/HF)	nc	↓	nc
Cpt1	nc	**NA**	↓	**NA**
Ppara	↓	**NA**	↓	**NA**
Mt copy number	nc	**NA**	↑	**NA**

In Chapter 2, maternal metformin treatment was hypothesized to reduce maternal-obesity-mediated priming of excess offspring adiposity, increased glucose tolerance, insulin resistance and NAFLD, dependent on the sex of the offspring. The data presented in this thesis demonstrate clear sexual dimorphism in the response to both metformin and maternal obesity, supporting this aspect of the hypothesis. Indeed, as summary Table 36 demonstrates, metformin in HFm females was predominantly protective against the adverse phenotypic features described in the hypothesis, whereas metformin in HFm males had minimal beneficial effects. Full data are discussed in detail below.

8.2.2.1 Effect of maternal metformin on adult female HFm offspring whole-body physiology

In female HFm/C offspring, metformin reduced body weight, adiposity and improved glucose tolerance. In HFm/HF females, the drug did not significantly reduce adiposity, but did slightly reduce body weight and dramatically improved measures of glucose and insulin sensitivity.

The lack of improvement in glucose homeostasis parameters in HFm/C females is perhaps less surprising when the lack of maternal HF priming is taken into account; FBG concentration was identical between HF/C and C/C groups (confirming the results reported by Bruce *et al.*[70]), and measures of fasting plasma insulin and HOMA-IR were not statistically different from those of C/C animals, giving little margin for metformin to improve these measurements. Salomaki *et al*. reported similar results in offspring of both genders, showing that glucose tolerance after 5–6 weeks of HF diet was impaired in offspring of HF dams but not in offspring of HF dams given metformin[255].

Adiposity was reduced in HFm/C females compared with the untreated HF/C offspring, suggesting that metformin alters energy homeostasis in some manner. How this is achieved can only be

speculated on, as measurement of energy expenditure and food intake, although attempted, did not produce interpretable data. However, maternal obesity promotes obesity in adult offspring via a number of pathways, and metformin treatment may alter one or more of them. Offspring of obese dams develop hyperphagia[350], increasing energy intake. There have also been suggestions that maternal obesity reduces offspring resting energy expenditure[460].

The data reported in this thesis show metformin exerting no substantial effect on degree of adiposity in HFm/HF animals, presumably due to the lengthy postnatal HF diet overwhelming the protective effects seen in HFm/C offspring. However, metformin clearly exerts a beneficial effect on HFm/HF glucose homeostasis, suggesting that degree of adiposity is decoupled from glucose and insulin sensitivity in these animals. Although expansion of adipose tissue in humans and animal models is typically correlated with decreased insulin sensitivity and increased glucose intolerance, expansion of different adipose tissue depots is associated with different metabolic responses[319]. In humans and mice, greater levels of visceral adipose tissue are associated with increased insulin resistance compared with expanded subcutaneous adipose tissue[15]. In the mouse model reported in this thesis, although total visceral adipose tissue was reduced in HFm/HF females, the ratio of visceral to subcutaneous fat was unchanged, suggesting the beneficial effect of metformin on whole-body glucose homeostasis in HFm/HF females is probably unrelated to the relative adipose tissue depot masses. However, adipose tissue was not characterized in this study and thus it is not possible to comment on indicators of adipose tissue insulin sensitivity or expression of adipokines, which can affect systemic insulin sensitivity positively (in the case of adiponectin) and negatively (in the case of inflammatory molecules including TNF and IL-6)[461]. Plasma levels of TNF and IL-6 were not significantly altered by metformin treatment, and levels of IL-10, which exerts anti-inflammatory effects, were reduced by metformin irrespective of maternal diet. Thus, the reduced insulin resistance observed in HFm/HF females seems independent of measured markers of systemic inflammation.

8.2.2.2 Effect on maternal metformin treatment on adult HFm male offspring whole-body physiology

In contrast to the broadly beneficial and protective effects of metformin in HFm female offspring, the effect of metformin in HFm male offspring was predominantly neutral. Although maternal HF diet did not have an independent effect on body weight or degree of adiposity, HF/HF males were markedly more obese than C/HF males, and metformin did not reduce fat mass percentage or body weight in HFm/HF offspring.

These data are somewhat in agreement with those reported by Salomaki *et al.*, who reported that although female offspring of obese dams treated with metformin gained less weight and fat

during postnatal HF feeding than female offspring of non-treated obese dams, there was no significant difference in weight gain during the postnatal HF diet phase between male offspring of metformin treated or untreated dams[255]. There were also no effects of maternal metformin in HFm males on glucose homeostasis, which was impaired in HF/HF offspring. Without determining fetal sex to stratify data reported in Chapter 4, mechanisms underlying this sexual dimorphism cannot be elucidated but only speculated upon. Data suggest that female sex hormones might underlie the improved insulin sensitivity seen in some female offspring of obese dams compared with male offspring[258], and it is conceivable that the interaction between this protection and maternal metformin treatment might be responsible for the protective effect of metformin in HFm/HF female offspring compared with HFm/HF males.

8.2.2.3 Effect of maternal metformin treatment on HFm offspring liver phenotype

On the basis that the maternal obesity model used primes development of NAFLD and NASH in offspring at 30 weeks of age[269], the liver was investigated as a candidate organ for mediating the beneficial effects of metformin in female HFm offspring. The liver is a central organ in whole-body energy homeostasis, and has a critical role in regulating glucose and insulin sensitivity. There is evidence that NAFLD, while recognized as the hepatic manifestation of the metabolic syndrome, independently contributes to development of whole-body insulin resistance[462]. Accumulation of lipotoxic lipid species, in particular DAG, is associated with increased hepatic insulin resistance, which manifests as a multitude of effects, including upregulated gluconeogenesis and hepatic DNL[61]. Other non-lipid mediators of hepatic insulin resistance have also been described, including increased activity of inflammatory pathways[463]. Thus, a protective effect of metformin on maternal-obesity-mediated priming of lipid accumulation or hepatic inflammation might underlie the beneficial effects on offspring glucose homeostasis, particularly those observed in HFm/HF females, which were independent of effects on adipose tissue mass or composition.

8.2.2.4 Metformin is generally hepatoprotective in HFm female offspring

Severity of NAFLD, which was increased by the independent effect of maternal diet in this data, as in other animal models of maternal obesity[70], was decreased by maternal metformin treatment in female offspring, with substantial effects observed in HFm/C vs HF/C animals. As discussed earlier, HFm/C females do not have markedly impaired glucose homeostasis compared with both HF/C and C/C animals, thereby suggesting that the improvements in NAFLD severity observed are unrelated to any alteration in glucose tolerance, fasting blood glucose or HOMA-IR. Planned comparison testing suggested that reduced inflammation subscore was the primary contributor to reduced NAS, and this finding was accompanied by modestly reduced hepatic expression of the pro-inflammatory genes *Ccl2* and *Tlr2*.

There are several putative mechanisms that might explain the action of metformin on hepatic inflammation. Firstly, whole-body adiposity was reduced in HFm/C offspring compared with HF/C animals. Although not measured in this study, this reduced level of adiposity might be associated with reduced expression of pro-inflammatory cytokines by adipose tissue. du Plessis *et al.* showed that expression of pro-inflammatory genes in visceral and subcutaneous adipose tissue could predict histological severity of NAFLD in patients[464], highlighting the inherent involvement of adipose tissue in the disease. Further characterization of markers of adipose tissue inflammation are required to support this hypothesis. Secondly, levels of measured hepatic lipid, although not significantly reduced, trended towards reductions in HFm/C vs HF/C offspring. In other animal models of NAFLD, free cholesterol levels increase in parallel with levels of CEs; levels of CE were significantly reduced in HFm/C vs HF/C females, suggesting that levels of free cholesterol, which is lipotoxic, pro-inflammatory and associated with development of NASH[65,465], might also be reduced.

Metformin might also prime alterations to the gut–liver axis. Obesity and NAFLD are associated with impaired gut barrier function, elevated blood LPS levels and activation of TLR4 and TLR9 in the liver, driving hepatic inflammation[466]. In humans, degree of dysbiosis is associated with the severity of NAFLD[467]. Moreover, obese individuals show increased impairment of the intestinal barrier that is partially restored with weight reduction, suggesting that weight reduction in this model, seen in HFm female offspring, might improve intestinal barrier function, and thereby hepatic inflammation[468]. Metformin also exerts specific effects on microbiota composition and functional capacity in humans[469], raising the possibility that 1) early-life colonization of offspring is altered by maternal metformin supplementation; and/or 2) metformin treatment protects against gut barrier dysfunction primed by maternal HF diet[400]. Analysis gut microbiota composition in female offspring is being carried out.

Finally, metformin might alter the programming of the hepatic inflammatory response, either directly, or by modulating the activity of pathways that synthesize pro-inflammatory and anti-inflammatory molecules. The hepatic ratio of n-3 to n-6 PUFAs was assessed in offspring as increased relative levels of anti-inflammatory n-3 PUFAs are probably beneficial in NAFLD, yet the proportions were unchanged in this model. Harris *et al.* showed that metformin reduced fetal hepatic IFNγ levels in a maternal obesity mouse model, although whether this effect persists into adulthood is unclear[256].

In HFm/HF female offspring, metformin did not reduce NAS, any individual NAS subscore or measured hepatic lipid accumulation, yet there was decreased expression of hepatic inflammatory markers. Thus, these offspring display dramatically improved glucose homeostasis,

characterized by improved FBG, glucose tolerance and insulin sensitivity, independent of any measured NAFLD-related liver parameter. This finding might be related to the relatively low sensitivity of histological inflammation assessment, or could reflect changes to an unmeasured aspect of the liver phenotype, for instance levels of free cholesterol, DAG or another pro-inflammatory pathway that converges on insulin receptor signalling. The reduced mRNA levels of *Ccl2* and *Opn* suggest a reduced inflammatory state in HFm/HF livers compared with HF/HF offspring, and this requires validation. HFm/HF females show both reduced FBG, which primarily reflects hepatic resistance to insulin and increased gluconeogenesis, and impaired glucose tolerance, which is typically the result of impaired peripheral insulin sensitivity[470]. It may be that metformin increases insulin sensitivity in peripheral tissue, such as skeletal muscle; in line with this hypothesis, Tong *et al.* showed that improved glucose tolerance in offspring of obese dams treated with metformin was associated with enhanced skeletal muscle AKT signalling, which is indicative of increased insulin sensitivity, and decreased numbers of intramuscular adipocytes[359].

The reduced levels of the profibrotic gene *Opn* in HFm/HF females are suggestive of reduced fibrosis progression in this group compared with HF/HF females. Levels of *OPN* are increased in patients with NAFLD, and patients with advanced fibrosis have more hepatic OPN protein than patients with less advanced fibrosis[401,471]. Moreover, neutralization of OPN levels in three different mouse models of liver fibrosis markedly reduced the fibrogenic response[402], suggesting a critical role for OPN in fibrosis progression and protective effects of metformin treatment in HFm/HF females. Fibrosis as assessed by Sirius red stain was unaltered between groups presented in this thesis, yet other studies have shown that αSMA deposition (another marker of liver fibrosis) in a CCl_4-induced mouse liver injury model (which presents fibrosis far quicker than diet-induced injury models) was significantly increased by day 3 after treatment, whereas Sirius-red-assessed liver fibrosis was only apparent by day 28[472]. Thus, molecular markers can serve as an indicator of fibrosis prior to histologically assessed fibrosis becoming apparent. Further examination of molecular markers of fibrosis directly downstream of *Opn* (αSMA and Col1a10) in HFm/HF females are required to validate this hypothesis[473]. Preliminary data for *Col1a1* shows that HF/HF females have increased hepatic expression relative to C/C and C/HF offspring, confirming the presence of increased fibrosis markers in the liver in this model. Expression of *Col1a1* in offspring of metformin treated mothers has not yet been assessed.

Finally, metformin dramatically improved indicators of capacity for fasting mitochondrial fatty acid oxidation in HFm/HF female offspring, as indicated by increased plasma β-hydroxybutyrate levels. These changes were independent of mitochondrial copy number variations, and mRNA expression of *Cpt1*, the gene encoding the rate-limiting enzyme for β-oxidation, and *Ppara*, which encodes the pro-fatty-acid-oxidation transcription factor PPARα. Whether metformin improves

oxidative metabolism by protecting against priming effects on the mitochondrial ETC (known to be dysfunctional in HF/HF animals in this model[70]) or broader mitochondrial dysfunction is unknown. Oxidative stress induced by mitochondrial dysfunction promotes synthesis of inflammatory cytokines that inhibit insulin signalling[474]. Salomaki *et al.* found that metformin treatment in obese pregnancy was associated with marked changes in expression of genes related to the ETC in male neonatal liver, ~ 1 week after stopping metformin treatment[255]. These results included reduced expression of *Ndufs4* (a component of ETC complex I), *Uqcrh* (a component of ETC complex II) and *Atp5c1* (a component of ATP synthase). On the basis that deletion of apoptosis inducing factor (AIF, associated with complex I) reduces oxidative phosphorylation and leads to improved insulin sensitivity and protection against metabolic disease in mice[475], Salomaki proposed that similar reductions in ETC protein transcript levels might similarly afford long-term protection against metabolic disease, although this hypothesis has not been validated. Whether these changes are persistent into adulthood is unclear.

In summary, female HFm/C offspring did not demonstrate improved markers of glucose homeostasis, probably because glucose homeostasis was only modestly impaired in this group. Total adiposity was reduced by metformin. Total hepatic lipid levels were not significantly reduced, yet markers of hepatic inflammation were reduced. This finding may be related to reduced hepatic inflammation, reduced production of hepatic lipotoxic species, or improvements to the insulin sensitivity of peripheral tissue. Genes encoding transcription factors driving hepatic lipid storage and DNL were downregulated, yet this was not associated with significantly decreased lipid levels. Fasting β-oxidation was not altered. These findings suggest a protective effect of metformin on offspring of HF dams that do not receive a postnatal HF diet.

In HFm/HF females, percentage body fat was not decreased compared with HF/HF females, and there were no changes in NAFLD severity or total hepatic lipid levels; however, markers of glucose homeostasis were dramatically improved, and suggest improved hepatic and peripheral insulin sensitivity. Markers of hepatic inflammation were reduced, providing a putative mechanism for increased insulin sensitivity and reduced gluconeogenesis. Capacity for β-oxidation was increased, suggesting improved mitochondrial oxidative metabolism, which is known to be defective in HF/HF animals. Mitochondrial dysfunction could underlie and exacerbate hepatic inflammation and insulin resistance in this group, whereas metformin treatment rescues these changes.

8.2.2.5 Effect of maternal metformin treatment on adult male HFm offspring liver is associated with altered lipid homeostasis

Whereas metformin treatment in female HFm offspring improved the liver phenotype, treatment in male HFm offspring was predominantly neutral. However, some concerning effects were

observed, particularly in the HFm/HF group. Compared with HF/HF males, HFm/HF males had markedly increased hepatic lipid accumulation, comprised largely of TAG. Interestingly, this finding was not associated with increased NAS, greater adiposity or impaired glucose homeostasis, suggesting a decoupling of hepatic TAG levels and hepatic insulin sensitivity or histologically assessed inflammation. Such a decoupling could be observed because TAG is a relatively inert lipid, compared with FFAs, ceramide, DAG and free cholesterol[476]. For instance, patients with familial hypobetalipoproteinaemia have high levels of hepatic TAG due to a VLDL TAG export defect; despite this excess ectopic TAG, they show the same hepatic insulin sensitivity as BMI-matched patients without intrahepatic TAG accumulation[477]. In addition, hepatic overexpression in mice of DGAT2, which promotes TAG synthesis, leads to greater hepatic steatosis but no effect on whole-body or hepatic insulin or glucose tolerance[49]. The mechanisms promoting increased TAG accumulation in HFm/HF males are unclear; DNL index was clearly upregulated in this group, and this could be accompanied by suppressed VLDL-mediated hepatic export, yet this parameter was not measured in this thesis. Fasting plasma β-hydroxybutyrate levels were suppressed relative to C/C animals, signifying an inability to oxidize hepatic fatty acids in both HF/HF and HFm/HF males. The same degree of suppression of fasting plasma β-hydroxybutyrate levels in HF/HF and HFm/HF animal suggests another mechanism is responsible for the disparate hepatic TAG levels in these groups.

The male HFm/C group did not substantially differ in any respect from the HF/C group, giving no indication of any effect of metformin in HF dams in male offspring not exposed to a postnatal HF diet. Thus, the increased TAG accumulation in HFm/HF vs HF/HF males is dependent on postnatal diet.

8.2.3 Long-term effects of metformin on Cm offspring of both sexes

Male and female offspring of lean dams given metformin had exacerbated metabolic dysfunction when exposed to a postnatal HF diet, and evidence of some primed effects persisting even when offspring were weaned onto a C diet (Table 36). In Chapter 2, the final hypothesis was that maternal metformin treatment in lean dams would exacerbate the detrimental effect of postnatal HF diet in offspring of lean dams, in a sex-dependent manner. The data described in Table 36 clearly demonstrate detrimental effects of maternal metformin in offspring of lean dams; although sex differences between males and females of Cm dams were less pronounced than those observed between males and females of HFm dams, males arguably had greater metabolic dysfunction, typified by greater increases in adiposity and glucose homeostasis impairments.

Cm/HF females were substantially heavier, had more adipose and a greater proportion of visceral adipose tissue. These findings were accompanied by greater glucose intolerance but not increased FBG, compared with C/HF offspring, suggesting metformin impairs peripheral glucose disposal without markedly affecting hepatic insulin resistance. Salomaki *et al.* did not find an effect of metformin in lean dams on female offspring glucose tolerance in their study, yet this might be related to differences in experimental protocol[253]. Critically, in this thesis, NAS was dramatically increased in Cm/HF females compared with C/HF offspring, a finding driven by increases in all individual NAS components. Hepatic steatosis area was significantly increased, although there was no statistically significant increase in measured hepatic lipids; unmeasured lipotoxic lipid species, such as free cholesterol, could contribute both to the observed hepatic steatosis and to the exacerbated NAFLD severity[65]. In accordance with increased steatosis, DNL was increased with concomitant trends towards increased DNL enzyme expression. Fasting β-hydroxybutyrate levels and hepatic expression of *Cpt1* were also decreased in Cm/HF females relative to C/HF females, providing another mechanistic explanation (that is, impaired fatty acid oxidation) that might promote hepatic lipid accumulation. The finding that mitochondrial copy number is increased in Cm/HF females is of uncertain importance. Increased mitochondrial copy number might be an effect primed early in life by metformin via its acute activation of AMPK; alternatively, increased mitochondrial copy number might be an adaptive response to impaired lipid oxidation or mitochondrial dysfunction[478]. Evidence reported in Chapter 4 demonstrated that metformin treatment upregulated genes related to mitochondrial biogenesis, *Tfam* and *Sirt1*, suggesting that these changes in mitochondrial copy number might begin early in life. This hypothesis is supported by the increased numbers of hepatic mitochondria in Cm/C offspring, which do not show lowered β-hydroxybutyrate levels or increased NAFLD severity or hepatic lipid accumulation; moreover, Tong *et al*. showed that maternal metformin rescued maternal-obesity-mediated decreases in offspring skeletal muscle mitochondrial DNA content[145].

Male Cm/HF offspring showed the same pattern of metabolic defects as female Cm/HF offspring. However, dysfunction was arguably greater in males; degree of adiposity was increased 1.7-fold in Cm/HF vs C/HF males, compared with a 1.13-fold increase in Cm/HF females vs C/HF females. Glucose homeostasis was similarly impaired, with metformin treatment exacerbating insulin resistance and increasing FBG and glucose intolerance in Cm/HF males compared with C/HF males, whereas metformin in the same group in female offspring only significantly impaired glucose tolerance.

NAS was strikingly increased in Cm/HF males, and was associated with increased levels of hepatic lipid. Increased hepatic DNL probably contributes to this increase, and the lack of increased fasting β-hydroxybutyrate levels, compared with C/HF males, suggests the absence of a

compensatory increase in hepatic fatty acid oxidation. These findings are supported by studies of metformin treatment in lean pregnancy and models of maternal undernutrition. The detrimental effects of metformin in lean dams in the study of Salomaki *et al.* were greatest in male offspring, who showed impaired glucose tolerance and elevated FBG in response to postnatal HF diet, whereas female offspring did not[253]. Males also showed decreased expression of *Glut4* in adipose tissue, which might contribute to the defects in glucose disposal observed in this model. Whether similar effects of metformin treatment are observed in peripheral tissue in Cm/HF males in this thesis is unclear, but such effects would contribute to the glucose intolerance observed in this group.

Fetal and adult offspring data in this model, combined with evidence from other studies, suggests that metformin treatment in lean dams might induce a state mimicking caloric restriction, typified by increased *Sirt1* expression; moreover, there is evidence from studies of maternal nutrient restriction during pregnancy that glucose homeostasis of adult male offspring is perturbed to a greater extent than female offspring[479,480]. Critically, human data also suggests that infants born small-for-gestational-age and who exhibit rapid postnatal growth are at increased risk of obesity and NAFLD[481].

8.3 Limitations

There are a number of limitations to the studies in this thesis. Firstly, although the nature of the model used (combining maternal obesity, maternal glucose intolerance and high-fat, high sucrose diet and/or metformin throughout gestation and lactation) recapitulates many of the features of human pregnancy, this design makes it impossible to separate the individual contributory mechanisms. Further studies examining the effect of metformin in obese but not insulin resistant dams are required to complement the results of the MOP and EMPOWaR trials. This approach is especially important given the evidence presented here that metformin is severely detrimental offspring in lean dams who have appropriate glucose homeostasis.

Given data showing marked sex differences in the response to metformin, stratification of data based on fetal sex is important; in addition, investigation of placental function, which has been linked to the manifestation of sexually dimorphic maternal programming effects, will need to be assessed. The early-life mechanisms behind metformin-mediated priming are unclear; in HF fetuses and neonates, data from other studies suggests metformin could exert numerous beneficial effects, including reducing inflammation, hepatic lipid accumulation and improving placental function, yet these parameters have not been measured[255,256,268].

Furthermore, the characterisation of adult offspring hepatic inflammation and fibrosis in this model requires expansion by examining more pathways and molecules in both males and females. mRNA levels of key pro-inflammatory cytokines were increased by maternal obesity and reduced by metformin treatment, but this requires validation by assessment of protein cytokine levels, quantification and functional characterization of hepatic lymphocytes and quantitative measures of hepatic fibrosis and/or hepatic stellate cell activation. Diet-induced NAFLD models using C57BL/6 mice typically do not develop overt, histologically observed fibrosis until later life, meaning clear priming effects on fibrosis mechanisms might only be observed in aged mice (up to 1 year of age)[482]; this is the rationale behind other mouse models of developmental priming assessing hepatic fibrosis using a 1-year end-of-study time point[483]. A reduction in hepatic *Opn* levels in HFm/HF females compared with HF/HF females is suggestive of a beneficial effect of metformin on fibrosis progression, yet this awaits confirmation in liver pathology, which is required to diagnose fibrosis in NASH in humans [25]. The choice of a 30-week end-of-study time point for the study of offspring in this thesis is a compromise between allowing the development of metabolic disease and studying treatment and diet effects before the phenotype becomes so severe as to obscure any protective effects of metformin. Studying offspring at time points earlier than 30 weeks would enable the temporal order of metabolic abnormalities to be discerned, and thus provide an indication of causative mechanisms. Conversely, including another 1-year time point would enable assessment of the effects of metformin on histological, clinically relevant hepatic fibrosis severity (rather than relying on markers of fibrosis such as *Opn*), and might also expose alterations in glucose homeostasis in HF/C and HFm/C offspring studied in this thesis, which did not show marked FBG or HOMA-IR abnormalities at 30 weeks of. However, addition of these time-points requires two more cohorts of mice and substantial time and expense.

Relatively large heterogeneity was observed within groups for many of the parameters assessed, which might have contributed to the lack of observed significance. Although every effort was made to ensure that every group contained as many litters-of-origin as possible to improve power (as the mixed model approach used does not assume the independence of offspring) and increase effective sample size (as the litter of origin is effectively the experimental unit), loss of animals meant that some groups did not have the desired number of different litters represented. For instance, the Cm/C female group was, through necessity, only derived from four litters, despite comprising eight offspring. Moreover, measurement of certain parameters was limited to only n = 5 per group due to time, logistical or resource constraints. For instance, the characterization of hepatic lipid composition and abundance was conducted with an n = 5 per group, giving limited power to detect differences in means between groups compared with the n = 5-10 per group for physiological measurements. Assuming sample independence, at 80% power an effect size of 1.8

(Cohen's *d* for the difference in means between HF/C vs HFm/C female offspring total hepatic lipid, which was not significant in planned comparison tests) would require a sample size of 6 per group to achieve significance of < 0.05 in a two-tailed t-test. Thus, it appears that some parameter measurements were underpowered. The heterogeneity observed could have many causes. Measurement inaccuracies could have contributed, although it is notable that measurements which removed human bias and error, such as body composition as assessed by μCT, showed marked heterogeneity (most strikingly in Figure 13f, Cm/HF males vs C/HF males). Thus, the biological response of the offspring to maternal and postnatal HF diet and metformin seems to be a substantial source of variance. Variance between dams in response to diet and metformin probably compounds these effects; for instance, dams showed quite marked heterogeneity in their glucose tolerance responses to HF diet (mean IPGTT area under the curve in HF dams without metformin 2,206 mmol/l min with a standard deviation of 570 mmol/l min). In future studies, dams could be 'admitted' to the study only if they responded in a human-analogous-manner to chronic obesogenic diet, such as by demonstrating obesity and impaired glucose tolerance/fasting blood glucose.

Another potential limitation in this study was the lack of blinding to the identities of offspring. Although the animal identities in the most subjective measurements (assessment of liver histology) were blinded, logistical reasons (such as having to provide visually distinct diets) precluded blinding for experiments conducted in live animals, and this may have led to subconscious bias.

Finally, it remains a possibility that the effects of metformin observed in this study are the result of metformin toxicity or adverse effects, rather than mediated by the drugs beneficial metabolic and anti-inflammatory properties. Metformin overdose can very rarely result in acute lactic acidosis in humans, characterized by nausea, vomiting and a high risk of death[484]. However, the dose measured in dam plasma and amniotic fluid was well within normal ranges seen in human pregnancy, and no behavioural changes or symptoms of toxicity were observed in dams.

8.4 Conclusion

Data presented in this thesis highlight substantial offspring metabolic priming effects resulting from maternal metformin treatment, which have relevance for the use of the drug in humans. Although use of the drug in obese, glucose-intolerant dams eating an HF diet is moderately protective against development of NAFLD and disrupted glucose homeostasis in female offspring, male offspring show markedly increased hepatic TAG storage. Conversely, use of the drug in lean dams results in substantially worsened offspring NAFLD, greater adiposity and impaired glucose

homeostasis, in both male and female offspring. The exact mechanisms by which metformin induces detrimental metabolic priming in offspring of lean dams is unclear, as numerous maternal factors (obesity, glucose intolerance, raised FBG, HF diet and systemic inflammation, amongst others) are different between lean and HF dams. Thus, it is impossible to say which maternal factor is required during pregnancy for metformin not to prime severe offspring metabolic dysfunction in response to a postnatal HF diet. As such, the data presented here caution against metformin use in obese pregnancy in humans until the causative mechanisms are elucidated.

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